Regulation of p53 and p73 Function by PCAF and Adenovirus E1B 55-kDa Oncoprotein

By

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TABLE OF CONTENTS

Chapter 2 Article 1

Yue Liu, April L. Colosimo, Xiangjiao Yang and Daiqing Liao (2000). Adenovirus E1B 55Kilodalton oncoprotein inhibits p53 acetylation by PCAF. Molecular and Cellular Biology 20(15) :5540-5553

Chapter 3 Article 2

Yue Liu, Lisa Y. Zhao, XiangJiao Yang and Daiqing Liao. PCAF acetylates p73 and serves as a coactivator of p73-mediated transactivation (submitted)

Chapter 4 Sub-project 3

Identification of the p53 and AdE1B 55K binding sites on PCAF

Introduction
Materials and Methods
Results

Chapter 5 Discussion and general conclusion

References

Acknowledgements

II. Table of illustrations

List of figures :

List of tables :

III. List of abbreviations and symbols

aa	amino acid
Ad	adenovirus
Ade	adenine
AdE1B, E1B	Adenovirus early region 1 B protein
AdE1A,E1A	Adenovirus early region 1 A protein
AdE4orf6, E4orf6	Adenovirus early region 4 open reading frame 6 protein
AD	activation domain
ADA2	Adenosine deaminase 2
ARF	Alternative reading frame
3-AT	3-aminotriazol
Arg,R	arginine
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia related
BD	binding domain
bp	base pair
BSA	bovine serum albumin
BZLF1	Epstein-Barr virus lytic-switch protein 1
c-Abl	human cellular homolog of the Abelson retrovirus oncogene
^{14}C	carbon 14
CBP	CREB binding protein
cDNA	complementary DNA
CDK	cyclin-dependent kinase
Chk	Csk homologous kinase
CKII	casein kinase II
cpm	count per minute
DBD	sequence-specific DNA binding domain
DMEM	Dulbecco modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAPK	DNA-dependent protein kinase
DTT	dithiothreitol
EBV	Epstein-Barr virus
EBNA-5	Epstein-Barr Virus Nuclear Antigen 5
EDTA	ethylene diamine tetra-acetic acid

FAT	factor acetylation
FBS	fetal bovine serum
Gal	galactose
His	histidine
hr	hour
HAT	histone acetylation
HBV	hepatitis B virus
HPV E6	human papillomavirus early region 6 protein
INK4a	cyclin D-CDK4 inhibitor a
IPTG	isopropylthiogalactoside
IR	ionizing radiation
kb	kilobase
kDa	kilodalton
luc	luciferase
lys, K	lysine
M	mole(s)
Mdm2	human homologue of the murine double-minute type 2
M.W.	molecular weight
min	minute
mCi	milliCurie
mg	milligram
ml	millilitre
mM	millimole(s)
mRNA	messenger ribonucleic acid
mu	mutant
ng	nanogram
nmol	nanomol
NP40	nonidet P-40
OD	optical density, oligomerization domain
PAGE	polyacrylamide gel electrophoresis
PCAF	p300/CBP associate factor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKR	protein kinase R
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
pX	protein X
PXXP	proline-x-x-proline motif

RD	regulatory domain
RNA	ribonucleic acid
rpm	rotation per minute
SAM	sterile alpha motif
SDS	sodium dodecyl sulphate
SV 40 T	simian virus 40 large T antigen
TAD	transactivation domain
TAE	Tris-acetate-EDTA
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
TSA	trichostatin A
UV	ultraviolet
µg	microgram
µl	microlitre
µM	micromole(s)
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-alactopyranoside

Abstract (French)

Le suppresseur de tumeur p53 joue un rôle critique dans la régulation de la croissance cellulaire, particulièrement en réponse à divers stress tels que les dommages à l'ADN ou l'infection virale. Lors d'une infection virale, p53 devient une cible des oncoprotéines des virus à ADN. L'inactivation de p53 amène les cellules à croître de manière incontrôlée et les virus à se répliquer. Il s'agit d'une étape clé de la transformation cellulaire induite par le virus. L'oncoprotéine E1B-55kDa (E1B) de l'adénovirus humain lie et inactive p53, prévenant ainsi la transcription des gènes sous son contrôle. La régulation des fonctions de p53 s'effectue surtout par des modifications post-traductionnelles dont l'acétylation, la phosphorylation, la sumoylation et la glycosylation. p53 peut être acétylé par p300/CBP et PCAF. Cette acétylation contribue à la régulation générale de l'activité de p53. Elle a pour effet de stabiliser p53, de stimuler sa liaison séquence spécifique à l'ADN et de réguler sa localisation nucléaire.

Afin d'adresser le mécanisme par lequel E1B inactive p53, nous avons étudié la possibilité que E1B interfère avec l'acétylation de p53 médiée par PCAF. Nous montrons que E1B inhibe spécifiquement l'acétylation de p53 par PCAF *in vivo* et *in vitro*, alors que l'acétylation des histones de même que l'auto-acétylation de PCAF ne sont pas affectées. De plus, la liaison à l'ADN de p53 est plus faible dans des cellules exprimant E1B. Le domaine central de PCAF, contenant la région HAT, lie une région près de l'extémité C-terminale de p53 comprenant le résidu Lys-320 acétylé spécifiquement par PCAF. Le bromodomaine de PCAF peut aussi lier E1B au domaine C-terminal. Nous montrons aussi que E1B nuit à l'interaction entre p53 et PCAF, suggérant que E1B

pourrait inhiber spécifiquement l'acétylation de p53 par PCAF en empêchant l'interaction enzyme-substrat. Récemment, un homologue de p53 nommé p73 a été identifié. Cette protéine ressemble à p53 à la fois par sa structure et sa fonction. Des études précédantes ont démontrées que plusieurs protéines cellulaires et virales impliquées dans le réseau de p53, telles que Mdm2, p300/CBP et E4orf6, sont aussi impliquées dans le réseau de p73. Ces protéines régulent p53 et p73 de manière similaire, mais non identique. D'un autre côté, d'autres protéines incluant les trois oncoprotéines virales majeures (l'antigène grand T de SV40, HPV E6 et Ad E1B) inactivent p53, mais non p73. Afin de mieux connaître le réseau de p73 et de comparer les ressemblances et les différences entre les voies de p53 et p73, nous avons étudié la possibilité que PCAF active aussi p73. Nous montrons que PCAF se lie à un domaine central de p73 que l'on retrouve dans toutes les isoformes de p73 et à une petite région, seulement présente à l'extrémité C-terminale de p73α. Cette région de p73α est acétylée par PCAF au résidu Lys-623 lors d'expériences *in vitro*. p73α est acétylé *in vivo*, alors que p73β ne l'est pas. Nous montrons aussi que PCAF stimule de manière significative la fonction de transactivation de p73α comme celle de p73β. Le domaine HAT de PCAF est nécessaire pour l'activation de la transcription via p73β. Nous avons aussi montré que la mutation K623 à R de p73α entraîne une réduction de son activité transcriptionnelle. Ces résultats suggèrent que PCAF sert de co-activateur transcriptionnel de p73. En accord avec la littérature, nous montrons que E1B n'arrive pas à inactiver la transcription dépendante de p73α et p73β ou encore inhibe la fonction de PCAF sur p73 comme c'est le cas pour p53.

Abstract (English)

The tumor suppressor protein p53 plays a critical role in regulating cell growth, particularly in response to various types of stresses such as DNA damage and viral infection. p53 is targeted by the oncoproteins of DNA viruses during infection. The inactivation of p53 leads to uncontrolled cell growth and viral replication. This is a key step in virus-induced cellular transformation. The E1B-55kDa (E1B) oncoprotein of human adenovirus binds to and inactivates p53, preventing the transcription of p53-responsive genes. The functions of p53 are regulated mainly at the level of post-translational modification. This includes acetylation, phosphorylation, sumoylation, and glycosylation. p53 can be acetylated by p300/CBP and PCAF. This is a key modification that contributes to the overall activity of p53. Acetylation stabilizes p53, stimulates sequence specific DNA-binding and regulates its nuclear localization.

In order to address the mechanism by which E1B inactivate p53, we studied whether E1B interferes with p53 acetylation by PCAF. We show that E1B specifically inhibits p53 acetylation by PCAF in vivo and in vitro, while acetylation of histones and PCAF autoacetylation is not affected. Furthermore, the DNA-binding activity of p53 is diminished in cells expressing the E1B proteins. The central domain of PCAF, containing the HAT region, binds to a region near the C-terminus of p53 encompassing Lys-320, the specific PCAF acetylation site. It also binds to the C-terminus of E1B through its bromodomain. We further show that E1B interferes with the interaction between p53 and

PCAF, thus suggesting that E1B might specifically inhibit PCAF acetylase function on p53 by preventing the enzyme-substrate interaction.

Recently, a homologue of p53, called p73, was identified. It resembles p53 both in structure and function. Previous studies have shown that some cellular and viral proteins involved in the p53 network, such as Mdm2, p300/CBP and E4orf6, are also involved in the p73 network. These proteins regulate p53 and p73 in similar but distinct ways. On the other hand, other proteins including the three main viral oncoproteins (SV40 large T antigen, HPV E6 and Ad E1B) which inactivate p53, fail to inactivate p73. In order to shed light on the p73 network and compare the similarities and differences between the p53 and p73 pathways, we studied whether PCAF also activates p73. We show that PCAF binds to a central domain of p73 that is shared by all p73 isoforms, and a small C-terminal region that is unique to p73α. This region of p73α is acetylated by PCAF in vitro at lysine 623. p73α, but not p73β, is acetylated in vivo. We further show that PCAF significantly stimulates both p73α and p73β transactivation function. The HAT domain of PCAF is required for stimulation of p73β-mediated transcription. We also demonstrate that mutation of p73α K623 to R leads to a reduction in its transcription activity. These data suggest that PCAF serves as a transcriptional coactivator of p73. Consistent with other published results, we show that E1B failed to inactivate p73α- and p73β-dependent transcription or to inhibit PCAF function on p73.

CHAPTER 1 INTRODUCTION

The objectives of this research are to study the mechanisms of regulation of the human tumor suppressor p53 and its family member p73 by the cellular transcriptional coactivator PCAF and the adenovirus E1B 55-kDa oncoprotein. The introduction covers: p53 and its regulation by cellular proteins, inactivation of p53 by viral oncoproteins, as well as p73 and its regulation.

1.1. Human tumor suppressor p53 and its regulation by cellular proteins

The p53 protein is a tumor suppressor protein whose mutations are widely found in various types of cancers. p53 exerts its tumor suppressor function mainly through regulating the transcription of its target genes, involved in the cell cycle arrest and apoptosis (el-Deiry et. al., 1993; Levine et. al., 1997). p53 is largely stimulated in response to a range of cellular stresses such as DNA damage, drugs and viral infection. Many signals that induce tumor development can activate p53, including carcinogen-induced DNA damage, telomere erosion, aberrant proliferative signals, hypoxia, and loss of adhesion or survival signals (Ko et. al., 1996; Vousden, 2000). Once stimulated, p53 activates the transcription of its responsive genes and results in the affected cells undergoing growth arrest or apoptosis. This allows repair of damage and protects the normal organism.

In addition to the importance of the transactivation activity of p53 in inducing cell cycle arrest and apoptosis, other functions of p53 might also play a role in the process. Previous studies have shown that p53 serves as a transcriptional repressor by utilizing deacetylases through its interaction with mSin3a (Murphy et. al., 1999). Although the requirements for p53 transcriptional repression ability are not well established, this activity correlates well with the ability of p53 to induce cell death and apoptosis. Furthermore, activities of p53 that are entirely independent of transcriptional regulation but which likely contribute to p53 function in inducing apoptosis have also been described. These include the ability of p53 to drive relocalization of death receptors like Fas from the Golgi apparatus to the cell surface (Bennett, 1998) and the function of p53 in the mitochondria (Donahue et. al, 2001; Moll et. al., 2001).

1.1.1. Structure and function relationship of p53

The p53 protein contains four main domains (Fig. 1): the N-terminal transactivation domain (TAD), the central sequence specific DNA-binding domain (DBD), the C-terminal oligomerization domain (OD) and the regulatory domain (RD). The TAD domain is a very crucial domain for p53 transactivation function because it contacts many transcription regulatory factors such as the components of the basic RNA polymerase II transcriptional machinery, TFIID, TFIIH and the transcriptional coactivator p300/CBP. Phosphorylation in this domain by the DNA-PK family member ATM (on serine 15) and ATR (on serine 37) enhances the transcriptional activity of p53. Many other kinases including JNK, p38, CK1 and CAK have also been shown to be capable of phosphorylating TAD. Although the physiological relevance associated with each of

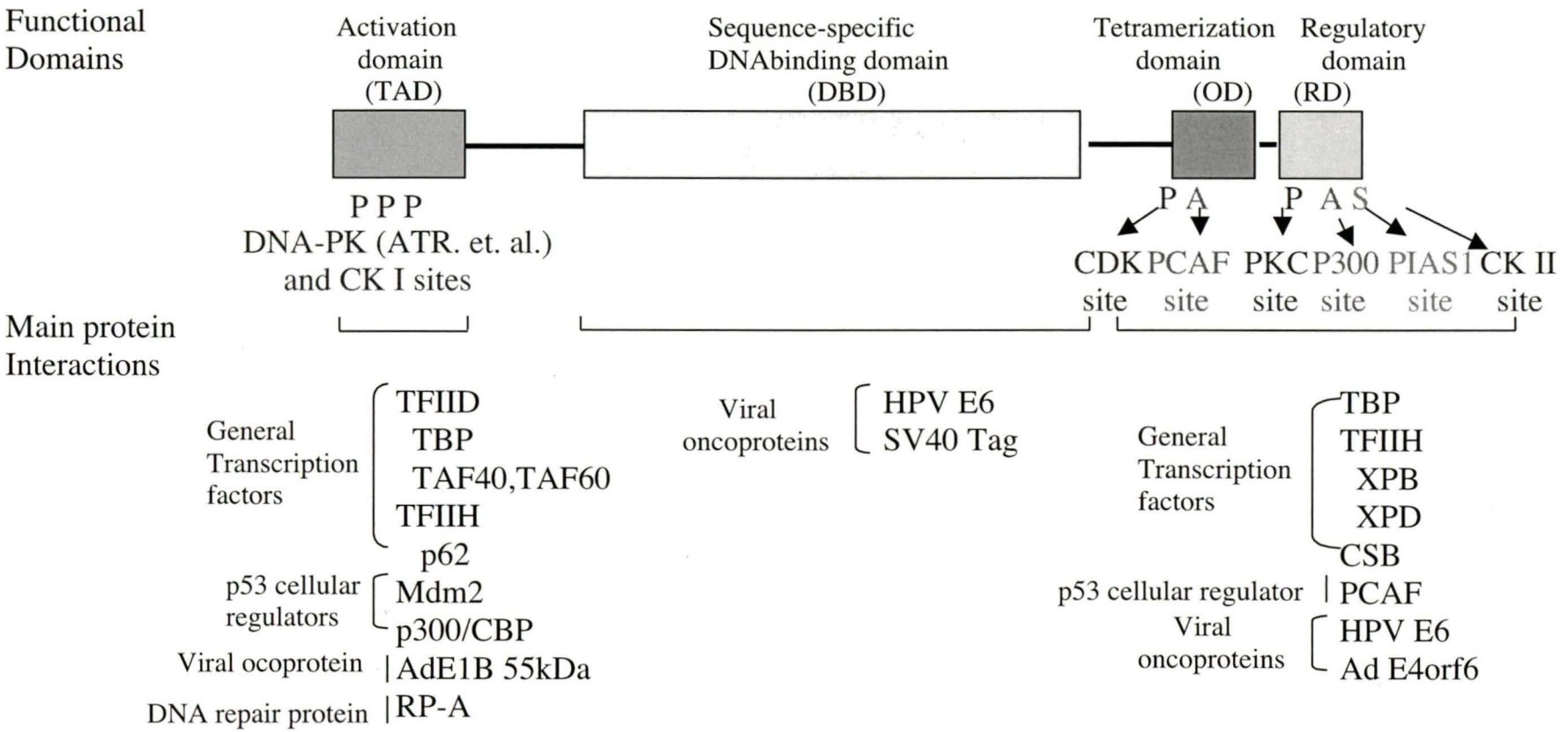

Fig 1. Structural organization of the p53 protein (modified from Ko and Prives, 1996). The four boxes represent the four functional domains of the p53 protein. The phosphorylation sites (P), acetylation sites (A) and the sumoylation site (S) were marked. The main protein interactions between different functional domains of p53 and other proteins such as the transcriptional factors, cellular, viral regulator proteins of p53 are shown at the bottom.

these observations has not yet been clarified, there is some suggestion that the N-terminal phosphorylation induced by DNA damage can regulate the interaction of p53 with p300/CBP, JNK and Mdm2 to regulate p53 transactivation function. The DBD recognizes and binds to a specific consensus sequence adjacent to p53 responsive genes. The OD domain enables p53 to form a tetramer, a structure that is required for p53 to bind to the target consensus DNA sequence. The RD domain regulates the transactivation activity of p53. When the specific sites within this domain are phosporylated, acetylated and/or sumoylated, p53 is stabilized and the DNA binding and transactivation activity of p53 may be stimulated (Ko et. al., 1996; Arrowsmith, 1999).

1.1.2. Regulation of p53 by cellular proteins

Many cellular and viral proteins regulate p53 function through direct interaction with the functional domains of p53 (Fig. 2). These proteins control p53 activity principally by regulating p53 protein stability, controling p53 subcellular localization and regulating post-translational modifications of p53 (Douglas et. al., 2001).

Inhibition of p53 function by Mdm2

Mdm2 interacts with p53 and inactivates it by 1) interfering with p53 interaction with p300/CBP, a transcriptional coactivator of p53, thereby preventing p300/CBP from stimulating p53-dependent transcription, and 2) serving as an E3 ubiquitin ligase specific for p53, hence targeting p53 to ubiquitin-dependent proteolysis. Thus, Mdm2 is a negative regulator of p53 (Wadgaonkar et. al., 1999; Honda et. al, 1997). Since the

Mdm2 gene is itself a p53 responsive gene, the regulation of p53 by Mdm2 is a feed-back auto-regulation process. A previous study demonstrated that the embryonic lethality in Mdm-2-deficient mice could be rescued by the deletion of the p53 gene. Elimination of the Mdm2-p53 auto-regulatory feedback loop is lethal (Leveillard et. al., 1998). This confirms the importance of the Mdm2-p53 feed back loop to control the concentration of p53 in cells. A recent study has shown that the Mdm2 family member MdmX also

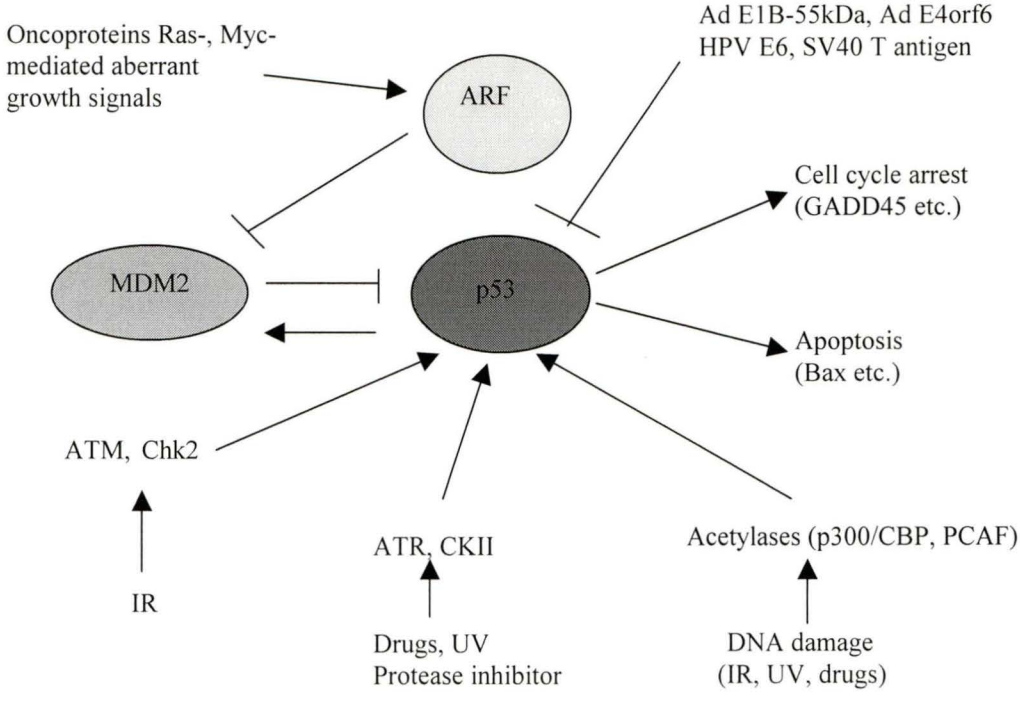

Fig. 2. Signaling pathways involving p53. Mdm2 inhibits p53 function. ARF abrogates Mdm2 function thus activating p53. Viral oncoproteins, Ad E1B-55kDa, SV40 T antigen and HPV E6 inactivate p53 function. In response to DNA damage or other external stresses, p53 can be activated through various pathways.

binds to p53 and inhibits p53 transactivation function. However, the mechanistic details of how MdmX affects p53 function are not yet clear (Wang et. al., 2001).

p53 inhibition by Mdm2 can be abrogated by ARF, a small polypeptide encoded at the INK4a locus. ARF relieves p53 inhibition by Mdm2 through two mechanisms: 1) It inhibits the p53-specific E3 ubiquitin ligase activity of Mdm2; 2) It alters the subcellular localization of Mdm2 by targeting both itself and Mdm2 to the nucleolus, thus releasing p53 from the inhibitory effect of an interaction with Mdm2 (Sherr et. al., 2000., Kamijo et. al., 1999). Previous studies have shown that mice lacking both ARF and p53 develop more multiple primary tumors of a wide range of types than mice lacking either gene alone. This demonstrates the importance of the ARF protein. Collectively, the ARF-Mdm2-p53 regulatory pathway is essential for the control of p53 concentration in cells (Kamijo et. al., 1999).

Activation of p53

The p53 network is normally 'off'. It is activated only when cells are under internal stresses. How can the network be triggered? Early research showed that DNA damage serves as the 'on' switch. Even a single break in a double-stranded DNA molecule is sufficient to trigger a rise in the levels of the p53 protein. Subsequent research demonstrated that the p53 response could be triggered by other signals, although it was difficult to know whether these other signals caused breaks in double-stranded DNA (Vogelstein et. al., 2000).

It has been confirmed recently that there are at least five independent pathways by which the p53 network can be activated (Fig. 2). The first pathway is triggered by DNA damage, such as caused by ionizing radiation (IR). In this pathway, the activation of the

p53 network is mainly dependent on two protein kinases, ATM (for ataxia telangiectasia mutated, named after a disease in which this enzyme is mutated) and Chk2. ATM is stimulated by double-strand breaks, and Chk2 is in turn stimulated by ATM (Chehab et. al, 1999 and 2000; Pandita et. al, 2000; Sherr et. al., 2000). The second pathway is stimulated by aberrant growth signals, such as those resulting from the expression of the oncogenes Ras or Myc. In this pathway, activation of the p53 network depends on the ARF protein, which abrogates the inactivation of p53 by Mdm2 and thus stabilizes p53 (Bringold et. al., 2000). The third pathway is induced by a wide range of chemotherapeutic drugs, ultraviolet light (UV), and protein-kinase inhibitors. This pathway depends on kinases called ATR (ataxia telangiectasia related) and casein kinase II (Lakin et. al, 1999; Hoffmann et. al., 1998). The fourth pathway is also induced by DNA damage such as IR and UV. This pathway depends on acetylases p300/CBP and PCAF (Liu et. al, 1999; Sakaguchi et al., 1998; Gu et. al., 1997). All these pathways inhibit the degradation of p53, thus stabilizing p53. The stabilization of p53 allows it to carry out its major function, binding to particular DNA sequences and activating the transcription of adjacent target genes. These genes will lead to cell death or to the inhibition of cell division.

Several novel modifications of p53 have been described recently, including sumoylation, glycosylation and poly ADP-ribosylation (Kwek et. al, 2001; Fiordaliso et. al., 2001; Smith et. al., 1999). All these modifications stabilize p53 and may reveal new pathways in activating the p53 network. In the following section, the DNA damage-PCAF-p53 pathway will be discussed in detail.

1.1.3. p53 activation by PCAF

In response to DNA damage, such as IR and UV, p53 can be activated by p300/CBP- and PCAF-mediated protein acetylation. p53 acetylation is believed to stabilize p53, stimulate p53 sequence specific DNA-binding activity and p53 coactivator recruitment ability, thus activate p53 transactivation function.

What is PCAF?

PCAF was initially identified as a p300/CBP associated factor (Yang et. al., 1996). Like p300/CBP, PCAF serves as an acetylase and transcriptional coactivator of many transcription activators such as MyoD and E2F1 (Sartorelli et. al., 1999; Martinez-Balbas et. al., 2000; Muth et. al., 2001.). The PCAF protein exerts its function through three main activities: the histone acetylation activity (HAT), the transcription factor acetylation activity (FAT) and the activator binding activity. PCAF acetylates histones, primarily on lysine-14 of histone H3, and weakly on lysine-8 of histone H4 (Schiltz and Nakatani 2000). Once recruited to the target DNA through its interaction with DNA bound transcription factors, PCAF might acetylate the nucleosomal histones thus promoting access of the RNA polymerase II transcriptional machinery to the adjacent DNA leading to the activation of transcription. Therefore, the HAT function of PCAF is thought to play a role in PCAF coactivator function. PCAF utilizes its FAT activity to acetylate transcription factors such as p53 and MyoD. Acetylation of these factors leads to enhanced protein stability, DNA binding activity and protein-protein interaction (Liu et. al., 1999; Sartorelli et. al., 1999). The activator binding function enables PCAF to be recruited to the DNA bound transcription activators, some of which are its substrates, to

co-activate the transcription of the adjacent gene and serves as a linker between its bound transcriptional activator and the PCAF complex. The PCAF complex contains more than 20 polypeptides. It might play a role in recognizing DNA damage and transducing a signal to PCAF and/or p300 to stimulate their acetylase activities (Schiltz and Nakatani 2000).

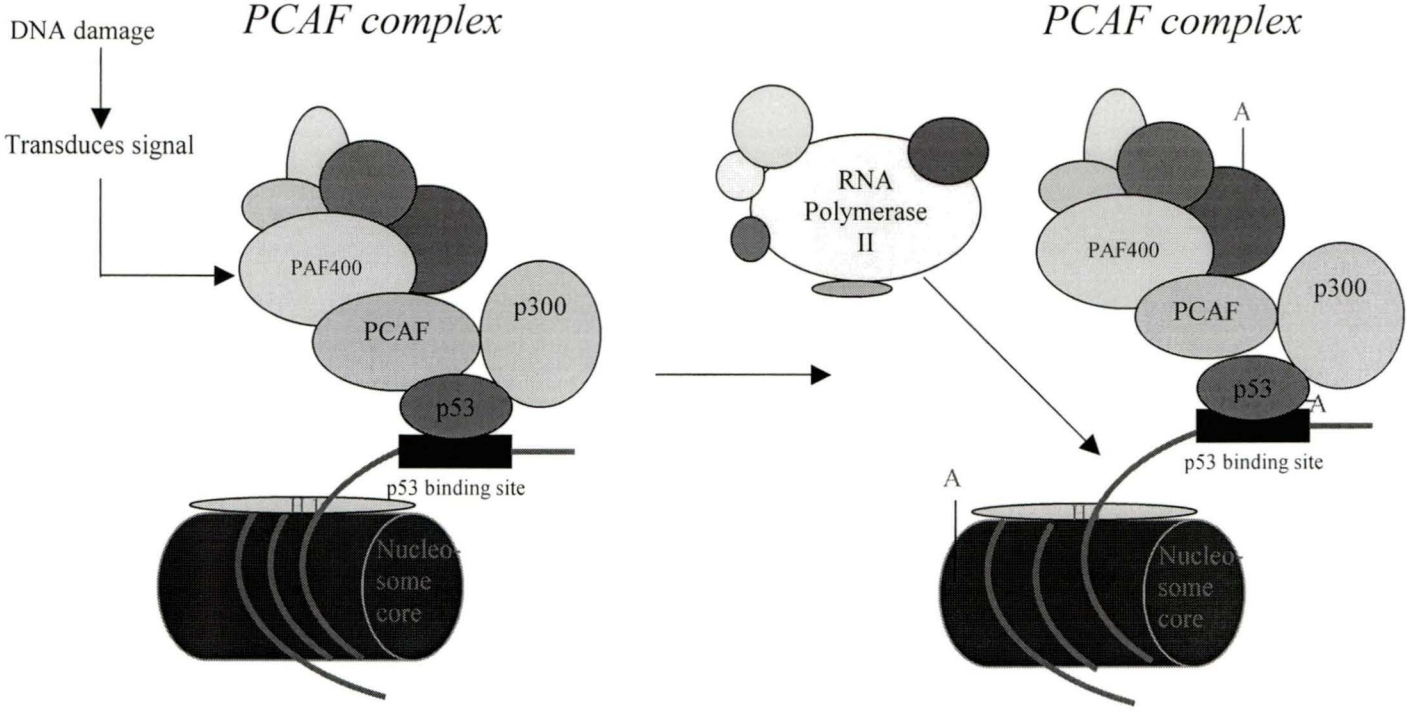

Fig. 3. Possible model for PCAF activation of p53. Once recruited to DNA-bound p53, PCAF might form a complex with p300 and serve as a bridge between p53 and the PCAF complex. In response to DNA damage, the damage signal might be transduced from PAF400 to PCAF and PCAF-bound p300 thus activating PCAF and p300. Hence, PCAF and p300 could acetylate p53 and activate p53 transactivation function.

How does PCAF activate p53?

The full function of p53 requires both PCAF and p300. The two factors form a complex in regulating p53 function. Both p300/CBP and PCAF bind to and acetylate p53 (Fig. 3).

However, they bind to different regions of p53 and acetylate p53 on different sites. While p300 binds to the N-terminal TAD of p53, PCAF interacts with the C-terminal OD of p53. Both p300 and PCAF acetylate p53 on its C-terminus. The sites acetylated by p300 are lysine 373 and 382, located in the RD domain, and the acetylation site by PCAF is lysine 320, sitting in the RD domain of p53 (Liu et. al., 1999; Liu et. al., 2000) . While p300 stimulates p53-dependent transcription in vitro in a transient reporter assay, PCAF fails to do so (unpublished data from our lab). Therefore, PCAF, p300/CBP may regulate p53 cooperatively but in a distinct way or to a varying extent.

Based on these studies, a possible model for the regulation of p53 by PCAF is shown in figure 3. Once recruited to the DNA-bound p53, PCAF might form a complex with p300 and serve as a bridge between p53 and the PCAF complex. In response to DNA damage, the damage signal could be transduced from a component of PCAF complex, PAF400 to PCAF and PCAF-bound p300 thus activating PCAF and p300. The activated PCAF and p300 acetylate p53 thus activating the p53 transactivation function. Besides, PCAF might also play a role in acetylating the nucleosomal histones, thereby promoting the access of the RNA polymerase II machinery to the adjacent DNA to activate transcription.

Although p53 acetylation is believed to play an important role in enhancing p53 DNA binding activity, there are some different views. Recent reports indicate that although p53 acetylated by p300 binds to a short, naked double-strand DNA better than non-acetylated p53, acetylation had no obvious effect on the ability of p53 to bind to chromatin and to activate transcription from a nucleosomal template in vitro. Aside from this, while the

binding of p53 to a 25 bp double-strand substrate was increased 12-fold by acetylation, the binding activity of p53 to a long DNA template of 160bp is increased only 1.5-fold by acetylation (Espinosa and Emerson. 2001). Therefore, whether acetylation of p53 increases p53 DNA binding ability is not yet clear. Another recent report demonstrated that p53 acetylation enhances its association with transcriptional coactivators such as CBP and TRRAP, results in increased histone acetylation and leads to activation of p53-dependent transcription (Barlev et al., 2001). Thus, one critical function of acetylation might be to promote p53 coactivator recruitment and histone acetylation. Acetylation also plays important roles in stabilizing p53 (Liu et. al., 1999; Gu et. al, 1997) and regulating p53 nuclear retention (unpublished data from our lab).

Interestingly, phosphorylation of p53 within the N-terminus by kinases such as ATM or ATR enhances p300 and PCAF binding and C-terminal acetylation, revealing a complex pathway in which multiple codependent modifications of p53 may be necessary for full activation (Kouzarides, 2000; Sakaguchi et al.,1998). Inhibition of acetylation by the action of either histone deacetylases or MDM2 results in a reduction in the transcriptional activity of p53.

An additional player in the pathway leading to p53 acetylation was recently reported as PML. PML would facilitate the recruitment of p53 to nuclear bodies. PML functions as a coactivator for p53, and the formation of PML-p53-CBP complexes in response to Ras correlates with the acetylation of p53 and the stimulation of p53 transactivation (Pearson et. al., 2000).

1.2. Inactivation of p53 by viral oncoproteins

p53 function is also regulated by viral proteins. A key step associated with the ability of small DNA tumor viruses to induce cell transformation is p53 inactivation. These viruses develop specific strategies to inactivate p53. This is necessary because induction of unscheduled DNA synthesis by viral infection appears to activate p53 and p53-dependent apoptosis. All small DNA tumor viruses must induce DNA synthesis in quiescent host cells to replicate viral DNA. Simian virus 40 (SV40), polyomavirus, human papillomavirus (HPV) and adenoviruses (Ad) have been the best characterized. After infecting the cells of their hosts, these viruses produce proteins that bind to and inhibit the retinoblastoma protein (pRb) and p53 thus encouraging cells to become cancerous.

1.2.1. Several strategies adopted by small DNA viruses to inactivate p53

Different DNA tumor viruses have adopted different strategies to inhibit the lethal effects of p53. SV40 large T antigen binds directly to the p53 DNA-binding region and blocks its interaction with p53-specific promoter elements (Sachsenmeier et. al., 2001). The E6 protein of the high-risk group of HPV associated with cervical carcinoma binds to p53 and targets p53 in ubiquitin-dependent degradation process. E6 is believed to serve as a bridge between p53 and E6-AP, an ubiquitin-protein ligase, thereby facilitating the rapid degradation of p53 (McGlennen et. al., 2000.). The hepatitis B virus (HBV) pX protein interacts with p53, diminishing both the DNA binding and the transactivation activities of p53 (Murakami 1999). Epstein-Barr virus (EBV) encodes two proteins that bind to and inactivate p53. One is EBNA-5, which appears to interact with both pRb and p53 and

may function to disrupt the normal cell cycle in a fashion similar to that of the DNA tumor virus oncoproteins described above (Pokrovskaja et. al., 1999). The other is BZLF1, which binds to the carboxyl terminus of p53 and blocks transactivation (Dreyfus et. al., 2000). The evolution of such widespread strategies clearly demonstrates the importance of p53 in the host cell defense against virus infection and the need for viruses to eliminate p53 function.

1.2.2. Inactivation of p53 by adenovirus oncoproteins

Adenoviruses were first isolated and characterized as distinct viral agents in 1953. Today, more than 100 members of the adenovirus family have been identified which infect a wide range of mammalian and avian hosts. All of these viruses contain a linear, double-stranded DNA genome encapsidated in an icosahedral protein shell measuring 70 to 100 nm in diameter. The Adenovirus chromosome carries five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and Iva2), and one late unit (major late) which is processed to generate five families of late mRNAs. The two oncoproteins encoded by the E1A and E1B gene mainly contribute to adenovirus transformation function (Barbanti-Brodano 1995). The full transformation function of Ad requires both E1A and E1B oncoproteins (Fig. 4).

Role of E1A in adenovirus induced transformation

The main function of E1A is to inactivate the pRb pathway which causes the deregulation of the cell cycle. pRb is a tumor suppressor protein whose loss or alteration relates to the development of a number of human neoplasias. In normal dividing cells, pRb phosphorylation is tightly regulated during different phases of the cell cycle. During the

late G1 phase, pRb is hyper phosphorylated and remains in this phosphorylation state through S, G2, and much of M. During G0 and much of G1, pRb becomes un- or hypo phosphorylated. Repeated hyper-phosphorylation of pRb in mid-late G1 again removes the cell cycle block associated with un- or hypo phosphorylated pRb. pRb phosphorylation is solely regulated by complexes of cyclins and cyclin dependent kinases

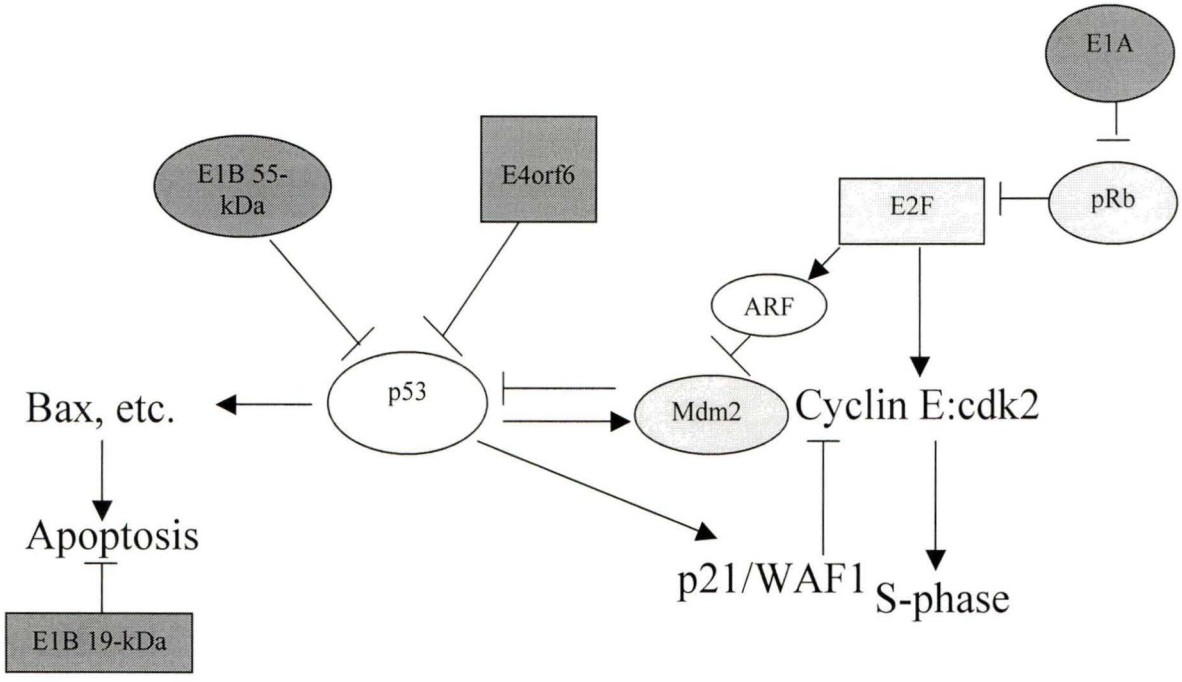

Fig 4. Role of Adenovirus oncoproteins in Ad induced transformation. While E1A inhibit the pRb pathway. E1B 55-kDa inactivate p53, form a complex with E4orf6 and other cellular proteins to target p53 to ubiquitin dependent protein degradation. E1B 19-kDa plays a role in inhibiting Ad induced apoptosis.

(CDKs). The un- or hypo phosphorylated pRb blocks progression into late G1, the step that precedes DNA synthesis and replication. Thus, binding of E1A to un- or hypo phosphorylated pRb will remove this block and lead to continuous cellular DNA synthesis. On the other hand, E1A protein dissociates the E2F-pRb complex, causing the release of E2F and the formation of an E1A-pRb complex. By liberating E2F

transcriptional factors and preventing them from re-associating with pRb, E1A disrupts the normal periodicity of the cell cycle and overcomes normal growth-restraining mechanisms, allowing the cell to undergo continuous proliferation (Parreno et. al., 2001; Barbanti-Brodano 1995).

In addition to inactivating pRb, E1A also inhibits the functions of other cellular proteins. These actions of E1A might also play a role in stimulating Ad-induced cell transformation. For example, E1A inhibits the activity of p300/CBP, which may result in a block in differentiation (Zhang et. al., 2000). The zinc finger of CR3 of E1A was shown to bind to TBP and other transcription factors such as dTAF 110 and dTAF 250. This adaptor function of E1A is also helpful for its function as a trans-activator (Barbanti-Brodano 1995).

Inactivation of p53 by E1B-55kDa

While E1A is required to inactivate the pRb pathway, E1B is required to inactivate the p53 pathway. During infection, the E1B promoter is transactivated by the E1A gene product. The E1B region transcribes two major overlapping RNAs of 13S and 22S, which encode unrelated proteins of 19 and 55 kDa, both of which are important for efficient viral replication. E1B 19-kDa protein can block E1A-induced apoptosis through an unknown mechanism, and prevent the pronounced degradation of both viral and cellular DNAs (Barbanti-Brodano 1995).

The E1B 55-kDa protein plays an important role during human adenovirus 2/5 productive infection. In the early phase of the viral infection, E1B 55-kDa binds to and inactivates the tumor suppressor protein p53, allowing efficient replication of the virus. During the late phase of infection, E1B 55-kDa is necessary to shut off cellular DNA synthesis and, in physical association with another early protein E4orf6, is important for efficient nucleo-cytoplasmic transport and translation of late viral mRNAs (Dix et al., 2000; Barbanti-Brodano 1995). Recent results with E1B 55-kDa protein in Dr. Arnord J Berk's lab showed that at 14 h post infection with Ad5 in HeLa cells, E1B 55-kDa is in a complex with E4orf6, cullin 5, and Elongin B and C. This complex can polyubiquitinate p53 in vitro (A. Berk, personal communication). This discovery is a very big clue on the mechanism by which E1B 55-kDa regulates p53 and succeeds in stimulating late viral gene expression. This complex may be targeting other cellular proteins for ubiquitination in addition to p53. This may affect late gene expression because of the degradation of some target cellular protein(s), or possibly the activation of some specific cellular protein(s) by addition of small proteins related to ubiquitin such as SUMO and Nedd8.

The central function of the E1B 55-kDa protein in Ad-induced transformation is to interfere with the normal activity of the cellular tumor suppressor protein p53. However, the mechanistic details of how E1B 55-kDa inactivates p53 are not yet completely clear. There are several possible scenarios based on previous studies. 1) The E1B 55-kDa protein repression domain may be tethered to the transcriptional machinery through its interaction with DNA-bound p53. In agreement with this, it was shown previously that Ad2 E1B 55-kDa protein has a generalized transcriptional repression activity, and

electrophoretic mobility shift assays indicated that E1B 55-kDa protein can supershift a p53-DNA complex. Furthermore, purified Ad2 E1B 55-kDa protein can specifically suppress p53 transactivation function in an in vitro transcription assay (Martin et. al., 1999). 2) E1B 55-kDa protein might interact with histone deacetylases and bring them to chromatin through interaction with E1B 55kDa and DNA-bound p53 in a manner similar to that of a number of known transcription repressors. 3) The direct interaction between E1B 55-kDa and TAD domain of p53 might inhibit the TAD function by preventing it from interacting with other transcriptional factors. 4) The E1B 55-kDa oncoprotein might inhibit post-translational modifications of p53, as covalent modifications of p53 such as phosphorylation and acetylation play important roles in activating p53. We set out to study whether E1B 55-kDa affects p53 acetylation by PCAF.

In chapter 2, we show that E1B 55-kDa protein from both Ad2 and Ad12 inhibits acetylation of p53 by PCAF in vitro and in vivo. In contrast E1B does not affect acetylation of histones by PCAF or PCAF auto-acetylation. Moreover, the DNA-binding activity of p53 in cells expressing E1B 55-kDa protein is greatly reduced. PCAF interacts with a region near the C-terminus of p53 as well as with both Ad2 and Ad12 E1B 55kDa proteins. In addition, E1B 55kDa protein appears to interfere with the physical interaction between PCAF and p53. These results suggest that abrogation of p53 acetylation by viral oncoproteins (such as the E1B 55kDa protein) inactivates p53 and thereby contributes to viral infection and cell transformation.

1.3. The p53 family member p73 and its regulation

For more than twenty years, p53 was thought to be the only gene of its kind. This view, which was widely accepted in the p53 field, has now been proven to be incorrect. Recently, two genes, termed p63 and p73 have been identified. They encode proteins resembling p53 in both structure and function. The identification of a p53 family of transcription factors has challenged us to understand the basis for their similarities and differences in terms of structure and functions, as well as their regulation and their mechanisms of activation.

1.3.1. Structure and expression of p73 gene

As seen in figure 5, like p53, p73 contains an N-terminal transactivation domain (TAD), a central sequence-specific DNA-binding domain (DBD) and a C-terminal oligomerization domain (OD). Unlike p53, p73 contains a possible second transactivation domain, a sterile alpha motif (SAM) and a small C-terminal region that might be a regulatory domain (RD). The SAM domain is a protein-protein interaction domain thought to be involved in the regulation of development. Both the SAM and RD are unique to p73α but not other isoforms of p73. A big difference between p53 and p73 is that while the p53 gene gives rise to only one protein product, the p73 gene produces over six proteins derived from alternative splicing. All these proteins contain the TAD, DBD and OD but have different truncated C-termini (Marin et. al., 1999; Chi and Ayed 1999; Arrowsmith 1999). The highest level of homology between p53 and p73 is reached in the DBD, which has 63% identity between p53 and p73. This indicates that the two proteins might bind to the same consensus DNA sequences and transactivate the same promoters. Indeed, p73

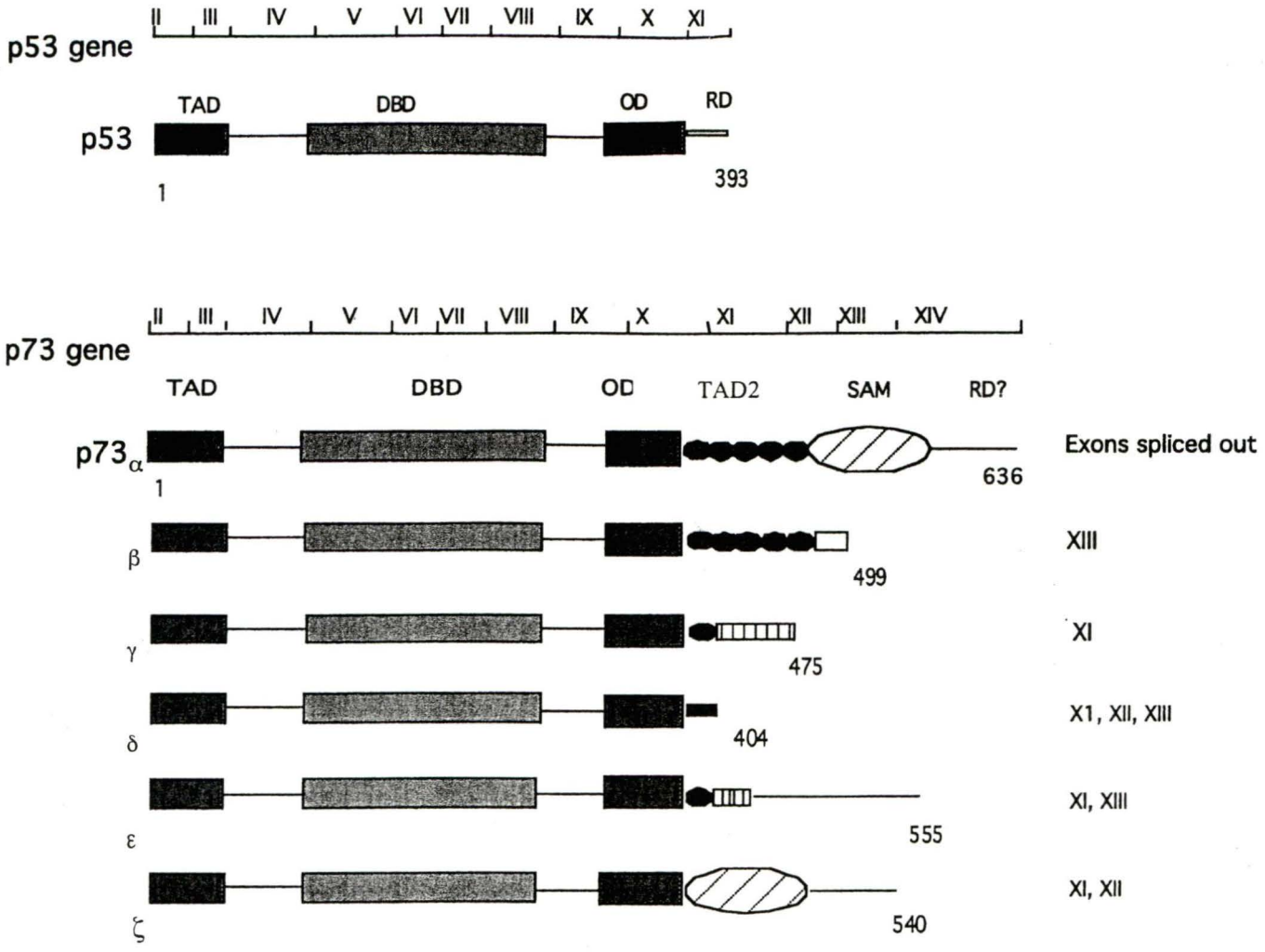

Fig. 5. Schematic representation of p73 isoforms (modified from Irwin and Kaelin 2001). All p73 isoforms contain the transactivation domain (TAD), sequence specific DNA binding domain (DBD) and oligomerization domain (OD). However, they have different C-termini derived from alternative splicing.

binds to the p53-specific consensus DNA sequence and activates the transcription of many p53 responsive genes involved in cell growth control (Irwin and Kaelin 2001). The high similarity of the OD between p53 and p73 suggests that members of the p53 family might form hetero-oligomers as well as homo-oligomers. However, yeast two-hybrid assays and co-immunoprecipitation experiments have shown that both p53 and p73 tend to form homo-oligomers rather than p53-p73 hetero-oligomers. Only some p53 dominant negative mutants (p53-

R175H and p53-R248H) can bind to p73 and inactivate its transactivation function (De Laurenzi and Melino 2000, Strano et. al., 2000).

1.3.2. Functional characteristics of p73

The main functions of p53 and p73 are compared in table 1. Like p53, p73 binds to the p53-specific consensus DNA sequence to transactivate many p53 target genes such as p21/WAF/Cip1, Mdm2 and bax (Irwin and Kaelin 2001; De Laurenzi and Melino 2000). Unlike p53, which is activated by ATR-, ATM- mediated phosphorylation or p300/CBP-, PCAF- mediated acetylation in response to DNA damage, p73 proteins undergo c-Abl mediated tyrosine phosphorylation when the cell is under external stresses (Gong et. al., 1999). While p53 is principally induced by the DNA damage caused by IR or UV treatment, p73 is mainly induced by the DNA damage caused by drugs such as cisplatin and doxorubicin. These reagents include Cisplatin, which crosslinks DNA, and taxol, an agent that stabilizes microtubules and prevents mitosis induced p73 accumulation (De Laurenzi and Melino 2000).

The high homology between p73 and p53, and the observation that p73 maps to chromosome 1p36.1, a region frequently deleted in several tumors has led to the suggestion that p73 might also be a tumor suppressor. However, unlike p53, mutations of which are frequently observed in various types of cancers, p73 mutations are rarely observed in tumors. Recent results showed that several p53 mutant proteins could associate with p73 and interfere with the transcriptional function of p73. The association between mutant p53 and p73 was detected in various tumor cells under physiological conditions. The Kaelin group even showed that the association between

	p53	p73
Genes responsive to regulation	p21/WAF/Cip1, Mdm2, GADD45 cyclin D1, cyclinG, bax, IGF-BP3, Wip1, EGFR, PCDNA, TGF-α, 14-3-3σ	p21/WAF/Cip1, Mdm2, GADD45 cyclinG, bax, IGF-BP3, VEGF, Ribonucleotide reductase
Upstream Signals	DNA-PK (ATR, ATM), p300/CBP, PCAF	C-Abl
Tumor suppressor function	Mutations are frequently observed in various types	Mutations are rarely observed in various tumors
Involvement in differentiation and development	No	Yes
Interactions with viral oncoproteins	AdE1B 55kDa, AdE4orf6, SV40 T-antigen, HPV E6	AdE4orf6
Interactions with cellular proteins	p300/CBP, PCAF, Mdm2, TFIID, TFIIH, TBP,TAF40,TAF60 etc.	Mdm2/MdmX, p300/CBP

Table 1. Comparison of p53 and p73 function. Like p53, p73 can activate many p53 responsive genes. Unlike p53, which is activated by DNA-PK-mediated phosphorylation and p300/CBP, and PCAF-mediated acetylation in response to DNA damage. p73 undergoes c-Abl-mediated tyrosine phosphorylation when the cell is under external stresses. While p53 mutations are widely observed in various types of cancers, p73 mutations are rarely observed in various tumors. p73 but not p53 plays an important role in cell differentiation and development. Many viral oncoproteins inactivating p53 failed to inactivate p73. While the signaling transduction pathway involving p53 is well studied, little is known about the p73 network. Recent studies indicated that Mdm2, p300/CBP which regulate p53 function also regulate p73. However, the two proteins apparently regulate p53 and p73 by somewhat different mechanisms.

mutant p53 and wild type p73 is governed by a common polymorphism at codon 72 of p53 that encodes Arg or Pro (Marin et. al., 2000). Thus, p73 transactivation function might be inactivated by mutant p53 in some types of cancers.

Studies with p73-/-mice showed that the ectopic expression of p73 in undifferentiated neuronal cells induces neurite outgrowth and expression of neuronal differentiation markers. Interestingly, in the same neuroblastoma cells, induction of differentiation by retinoids is accompanied by accumulation of p73. Moreover, transfection of dominant negative p73 in neuroblastoma cells abrogates the transactivation of the NCAM promoter induced by retinoids. Furthermore, in C2C12 skeletal muscle cells which were induced to differentiate in vitro, the level of p73 transcript is increased (Yang et. al., 2000; De Laurenzi and Melino 2000). These observations indicate that p73 might play a role in differentiation. Future work should tell which physiological signals recruit p73 to the developmental program.

1.3.3. Regulation of p73 by cellular proteins

While the transduction pathway involving p53 is well understood, little is known about the p73 network. Recent studies showed that two cellular proteins, Mdm2 and p300/CBP, which regulate p53 function, also regulate p73. However, the ways for the two proteins to regulate p53 and p73 are not completely identical.

Mdm2

As demonstrated in section 1.1, Mdm2 binds to p53, inactivates it, and targets p53 for ubiquitin-dependent protein degradation. Recent studies indicated that Mdm2 and its family member MdmX also bind to p73 (Zeng et al., 1999; Wang et. al., 2001). However, although Mdm2 binds to the p53 homologous domain in p73 N-terminus and inactivates p73 transactivation function, it failed to target p73 to ubiquitin-dependent proteolysis. Interestingly, p73 stability is also controlled by the proteosome, and treatment of cells with inhibitors of the proteosome results in the accumulation of p73 protein. Therefore, other unknown ways or proteins might play roles in directing p73 proteolysis (Zeng et. al., 1999). A recent study showed that p73 could transactivate the MDM2 promoter in p53-null cell lines, indicating a feed-back regulation loop between p73 and Mdm2 (Wang et. al., 2001).

p300/CBP

As a transcriptional coactivator and acetyltransferase of p53, p300/CBP binds to p53, acetylates p53 and hence stabilizes p53 and activates the transactivation activity of p53. Although p300/CBP also binds to p73, and the p73-p300 interaction can stimulate p73 dependent transcription and induce apoptosis, p300 interacts with p73 through a different domain than the one interacting with p53 (CH1 domain of p73 N-terminus and RD domain of p53 C-terminus). Furthermore, the acetylation function of p300 is not required for p300 to activate p73 (Zeng et. al., 2000 and 2001).

Given the homology in protein structure between p53 and p73 and the similarities of their functions, it is highly possible that many other factors involved in the p53 network are also part of the p73 network. In chapter 3, we have begun to address some of these issues by studying if PCAF is one of these factors involved in the p73 network. We showed that PCAF binds to a

central domain of p73 that is shared by all p73 isoforms. PCAF also interacts with a small C-terminal region that is unique to p73α. This small C-terminal region is acetylated by PCAF in vitro. We determined that K623 of p73α is a PCAF acetylation site. p73α but not p73β is also acetylated in vivo. We further showed that PCAF stimulates both p73α and p73β-dependent transcription and apoptosis. Although PCAF does not acetylate p73β in vivo, the PCAF HAT domain is required for PCAF to stimulate p73β-mediated transactivation. These data suggest that PCAF acetylates p73 and serves as a coactivator of p73-mediated transactivation. Therefore, unlike p53, of which the activation of transactivation function requires the acetylase function of both p300 and PCAF, the activation of p73 transactivation function might only need the acetylase function of PCAF. On the other hand, like p53, the full function of p73 may also require both p300 and PCAF. This study helps us to understand the basic mechanism by which p73 transactivates its responsive genes.

1.3.4. Interactions between p73 and viral oncoproteins

As demonstrated in section 1.2, oncoproteins encoded by certain DNA tumor viruses inhibit the function of p53. Several studies have examined the interaction of p73 with these viral proteins. These studies showed that the adenovirus E1B 55-kDa protein and the SV 40 T-antigen do not interact with p73α or p73β and failed to inhibit the transactivation function of p73 (Marin et. al., 1999; Wienzek et. al., 2000; Dobbelstein et. al., 1998). The HPV E6 protein has no effect on the stability of p73α or p73β, and overproduction of the p73β protein in E6-expressing cells leads to growth inhibition and apoptosis (Prabhu et. al., 1998). Though a recent report indicated that HPVE6 might bind to p73 and inactivate p73 function in vitro (Park et al., 2001), these

observations may probably indicate that the viral oncoproteins known to inactivate p53 do not inactivate p73.

The finding that the adenovirus E4orf6 protein inhibits the transactivation and the apoptotic functions of p73 α and β suggest that oncogenic viruses might inactivate p73 by using other strategies or other viral proteins. E4orf6 binds to a C-terminal region common of p73 α and β and, which might be corresponding identical to the E4orf6 binding domain on p53 (Higashino et. al., 1998). Moreover, the p73 protein, like p53, is up-regulated in cells infected with adenovirus, which suggests that inhibition of cellular p73 is also important for adenovirus infection. The E1A and E1B 55-kDa proteins, but not the E1B 19-kDa protein or E4orf6, increase the steady-state levels of both endogenous p73 and p53 (Steegenga et. al., 1999). E1A-induced accumulation of p53 protein is mediated by induction of p19ARF, which interferes with the Mdm2-mediated degradation of p53. However, since Mdm2 does not affect the protein stability of p73, thus the ARF-Mdm2-p73 regulation loop might not play a role in regulating p73 protein level. Therefore, the induction of p73 by E1A and E1B 55kDa is probably mediated by other unknown mechanisms. The inactivation of p73 by E4orf6 is probably crucial to the repression of cell death of adenovirus- infected cells.

In chapter 4, we confirmed that E1B 55-kDa not only failed to inactivate both p73α- and p73β-dependent transcription but also failed to affect PCAF function on stimulating p73 transcription. We also found that E1B 55-kDa, which inhibits p53 acetylation by PCAF, failed to inhibit p73 acetylation by PCAF. We discuss the possible mechanism of how E1B 55-kDa protein distinctly regulates p53 and p73 activity. We showed p53 binds to the central domain of PCAF, and E1B

binds to the bromodomain of PCAF. E1B can inhibit p53 acetylation catalyzed by PCAF lacking the bromodomain. Combined with that the E1B mutant lacking the PCAF binding domain can dissociate the p53-PCAF interaction (chapter 2), these data may suggest that p53-E1B interaction is sufficient to inhibit p53 acetylation by PCAF. The E1B-PCAF interaction may essentially play other roles. This may also be helpful to explain why E1B, which failed to interact with p73, failed to inactivate p73 and inhibit p73 activation by PCAF.

Based on these background knowledge, I choose the following questions as my Ph.D project: 1) Does E1B inhibit p53 acetylation by PCAF and if so, how? 2) Does PCAF acetylate p73 and serve as a coactivator of p73-mediated transactivation? 3) Does E1B affect PCAF function on p73? I think the research on these questions will help us to learn more about the p53 network and the emerging p73 network.

Chapter 2 Article 1

Yue Liu, April L. Colosimo, Xiangjiao Yang and Daiqing Liao (2000). Adenovirus E1B 55Kilodalton oncoprotein inhibits p53 acetylation by PCAF. Molecular and Cellular Biology 20(15) :5540-5553.

As the first author of this article, I did most of the experiments presented in this article. These include protein expression and purification (Fig. 1), in vitro and in vivo acetylation assay (Fig. 2 and 3), the EMSA assay (Fig. 4), the immunoprecipitation assay (Fig. 5) and some of the yeast two hybrid assays (Fig. 7, 8 and 9).

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Adenovirus E1B 55-Kilodalton Oncoprotein Inhibits p53 Acetylation by PCAF

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The adenovirus E1B 55-kDa protein binds to cellular tumor suppressor p53 and inactivates its transcriptional transactivation function. p53 transactivation activity is dependent upon its ability to bind to specific DNA sequences near the promoters of its target genes. It was shown recently that p53 is acetylated by transcriptional coactivators p300, CREB bidning protein (CBP), and PCAF and that acetylation of p53 by these proteins enhances p53 sequence-specific DNA binding. Here we show that the E1B 55-kDa protein specifically inhibits p53 acetylation by PCAF in vivo and in vitro, while acetylation of histones and PCAF autoacetylation is not affected. Furthermore, the DNA-binding activity of p53 is diminished in cells expressing the E1B 55-kDa protein. PCAF binds to the E1B 55-kDa protein and to a region near the C terminus of p53 encompassing Lys-320, the specific PCAF acetylation site. We further show that the E1B 55-kDa protein interferes with the physical interaction between PCAF and p53, suggesting that the E1B 55-kDa protein inhibits PCAF acetylase function on p53 by preventing enzyme-substrate interaction. These results underscore the importance of p53 acetylation for its function and suggest that inhibition of p53 acetylation by viral oncoproteins prevent its activation, thereby contributing to viral transformation.

The cellular tumor suppressor p53 exerts its tumor suppression functions largely by acting as a transcriptional transactivator. In response to a variety of stimuli, such as DNA damage and expression of cellular or viral oncoproteins, p53 is stabilized and binds to specific DNA sequences in the vicinity of the promoter of its target genes and activates their transcription. The genes activated by p53 include *p21 (* also called *WAF1* or *Cip1)* (cyclin-dependent kinase inhibitor), cyclin G, *GADD45, Mdm2,* and *Bax1* (apoptosis inducer). The products of these genes are implicated in regulation of cell cycle progression, DNA replication, and apoptosis (13, 25, 31).

Growth arrest or apoptosis imposed by p53 could severely hinder the replication of small DNA tumor viruses, as such replication requires host cells to enter the S phase. Thus, it is not surprising that a number of viral oncoproteins, such as the adenovirus (Ad) E1B 55-kDa protein, human papillomavirus (HPV) E6, and simian virus 40 large T antigen, bind to and repress the biological functions of p53 (30, 34, 49, 66). The E6 proteins of highly oncogenic HPV types 16 and 18 (HPV16 and HPV18) associate with p53 and target it for ubiquitination and subsequent degradation (51). Simian virus 40 large T antigen binds to the sequence-specific DNA binding domain of p53 (52, 56). This interaction interferes with sequence-specific DNA binding of p53 and therefore inhibits p53-mediated transcriptional transactivation (2, 10, 41).

Inhibition of p53 transactivation function is thought to be the key step in cell transformation induced by Ad (45, 69). The transforming function of Ad maps to the early region 1 (E1) of the 36-kb Ad genome (45). The E1 region encompasses two independent transcription units, E1A and E1B. E1A encodes two major polypeptides, 289R and 243R, whereas E1B transcript specifies two overlapping open reading frames which encode E1B 19-kDa and 55-kDa proteins. The E1A proteins bind to retinoblastoma protein pRb and inhibit its function in regulating cell cycle progression (11) and also appear to affect p53 functions (54). The E1B 19-kDa protein functions as an inhibitor of apoptosis (7, 36; reviewed in reference 46). The E1B 55-kDa protein suppresses p53 transactivation activity and also p53-mediated apoptosis (38, 55, 58, 69, 71). Both E1B proteins are required to fully transform cells in cooperation with E1A (3, 14, 60).

In Ad-transformed cells as well as in vitro, the E1B 55-kDa protein tightly associates with p53 (24, 48, 70, 72). Linker insertion mutagenesis of Ad type 2 (Ad2) E1B 55-kDa protein indicated that two regions around position H180 and between positions A262 and H326 are important for p53-E1B 55-kDa protein interaction (70). In a reciprocal study using in vitro immunoprecipitation (IP) assays, the amino-terminal 123 residues of murine p53 were shown to be responsible for binding to E1B 55-kDa protein (24). Several hydrophobic amino acid residues including Trp-23 and Pro-27 of human p53 are important for binding to the E1B 55-kDa protein, and these hydrophobic residues are also critical for p53 transactivation activity (33). These studies thus suggest that the interaction between E1B 55-kDa protein and p53 is important to inactivate p53 transactivation function. Further studies demonstrated that transcriptional repression function of E1B 55-kDa protein correlates with its ability to transform cells and that binding to p53 is necessary but not sufficient for transcriptional repression and transformation activities of the Ad2 E1B 55-kDa oncoprotein (71). Moreover, phosphorylation of three residues (Ser-490 and -491 and Thr-495) near the carboxyl terminus of Ad5 E1B 55-kDa protein is also required for transcriptional repression and transformation (57). The E1B 55-kDa protein from highly oncogenic Ad12 also represses p53 transactivation activity (55). It shares a high level of sequence identity with its Ad2 or Ad5 counterpart after position Lys-136 of the Ad12 protein, whereas sequence identity in the amino-

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terminal region up to residue Tyr-135 between Ad12 and Ad2 or Ad5 ranges from very weak to nonrecognizable (32). Since it is the conserved sequence within the E1B 55-kDa protein that is required for binding to p53, it is expected that Ad12 E1B 55-kDa protein might bind to p53 as well as does its Ad2 or Ad5 counterpart. However, previous studies using immunofluorescence microscopy and IP failed to detect interaction between Ad12 E1B 55-kDa protein and p53 (37, 61, 73). Nonetheless, these results do not prove that there is no interaction between p53 and Ad12 E1B 55-kDa protein; one possibility is that specific antibodies used in those studies could block the binding sites between p53 and E1B 55-kDa protein. Indeed, interaction between p53 and Ad12 E1B 55-kDa protein was detected in IP assays as well as in immunofluorescence microscopy using different antibodies (12, 16, 67).

How does E1B 55-kDa oncoprotein inhibit the p53-mediated transcriptional transactivation function? There are several possible scenarios. First, the E1B 55-kDa protein repression domain may be tethered to the transcriptional machinery through its interaction with DNA-bound p53. In agreement with this, it was shown previously that Ad2 E1B 55-kDa protein has a generalized transcriptional repression activity, and electrophoretic mobility shift assays indicated that E1B 55-kDa protein can supershift a p53-DNA complex (71). Furthermore, purified Ad2 E1B 55-kDa protein can specifically suppress p53 transactivation function in an in vitro transcription assay (39, 40). Second, E1B 55-kDa protein might interact with histone deacetylases and bring them to chromatin through interaction between E1B and DNA-bound p53 in a manner similar to that of a number of known transcription repressors (6, 27, 59). Third, the E1B 55-kDa oncoprotein might inhibit posttranslational modifications of p53, as covalent modifications of p53 such as phosphorylation and acetylation play important roles in activating p53 (42).

It has been shown recently that the acetylases p300, CREB binding protein (CBP), and PCAF acetylate p53 and enhance its sequence-specific DNA-binding activity (17, 35, 47). Furthermore, such acetylation is induced in response to DNA damage (35, 47), suggesting that this modification may represent a physiological response to activate p53; consequently, suppression of p53 acetylation may inhibit its sequence-specific DNA-binding activity and render it unable to transactivate transcription.

In this study, we show that E1B 55-kDa protein from both Ad2 and Ad12 inhibits acetylation of p53 by PCAF in vitro and in vivo, whereas it does not affect acetylation of histones by PCAF or its autoacetylation. Moreover, the DNA-binding activity of p53 in cells expressing E1B 55-kDa protein is greatly reduced. PCAF interacts with a region near the C terminus of p53 as well as with both Ad2 and Ad12 E1B 55-kDa proteins. In addition, E1B 55-kDa protein appears to interfere with the physical interaction between PCAF and p53. These results suggest that abrogation of p53 acetylation by viral oncoproteins such as the E1B 55-kDa protein inactivates p53 and thereby contributes to viral infection and cell transformation.

MATERIALS AND METHODS

Cell culture. Sf9 cells were maintained at 27°C in Grace's insect medium (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS), 1× lactalbumin hydrolysate, 1× yeastolate, and 0.1% pluronic F-48 (Gibco-BRL). A hybridoma cell line producing monoclonal antibody 2A6 against Ad2 or Ad5 E1B 55-kDa protein (a kind gift of Arnold Levine) was cultured in RPMI medium supplemented with 10% FBS. Monolayer cell lines, G401, G401-CC3, and 293, were cultured in Dulbecco's modified essential medium supplemented with 10% FBS, 100 U of penicillin, and 0.1 mg of streptomycin per ml. For culturing G401 and G401-CC3, hypoxanthine (15 μg/ml) and thymidine (10 μg/ml) were added to the medium. In addition, 250 μg of G418 per ml was added to the medium for growing G401-CC3. G401 is a rhabdoid kidney tumor cell line (65); G401-CC3 is a derivative of G401, which was stably transfected with a vector expressing Ad12 E1B 55-kDa protein (55). 293 was derived from human embryonic kidney cells by transformation with Ad5 DNA fragments, and this cell line expresses both E1A and E1B proteins (15).

Protein expression. The Ad E1B 55-kDa proteins were expressed in insect cells and in *Escherichia coli*. To construct recombinant baculovirus, a *Bam*HI-*Sal*I fragment containing the entire coding region of Ad2 or Ad12 E1B 55-kDa protein was excised from plasmid pGEM-T/Ad2 HA-E1B or pGEM-T/Ad12 HA-E1B, and this fragment was cloned into pFastBacHTb (Gibco-BRL) donor vector. To tag proteins with both the FLAG peptide and six His residues in the donor vector, an oligonucleotide containing codons for the 9 amino acid (aa) residues of the FLAG peptide was first inserted into *Bam*HI and *Eco*RI sites of pFastBac1, and then the *Bam*HI-*Hin*dIII fragment encompassing the FLAG coding sequence and multiple cloning sites of pFastBac1 was cloned into *Bam*HI and *Hin*dIII sites of pFastBacHTa to make the plasmid FLAG-pFastBacHTa. The human p53 coding region was cloned into the *Eco*RI site of FLAG-pFastBacHTa. Recombinant baculoviruses (bacmids) were generated by transforming DH10Bac competent cells (Gibco-BRL) with various donor plasmids. The DNA of these bacmids was isolated from *E. coli* and was transfected into Sf9 insect cells. Viruses were produced typically at 72 to 96 h posttransfection. To amplify the recombinant baculovirus, the supernatant of initial culture was used to further infect Sf9 cells. After one round of amplification, the viral titer was determined using plaque assay. We typically got a titer of $\sim 10^{10}$ PFU/ml. To determine optimal expression, Sf9 cells were infected with varying amounts of virus and harvested at different time points postinfection. The Ad2 and Ad12 E1B 55-kDa proteins with amino-terminal six-His tag and p53 with both six-His and FLAG tags at the amino terminus were expressed in the insect cells (see Fig. 1A).

The wild-type (WT) Ad12 E1B 55-kDa protein and its N-terminal portion were also expressed in *E. coli*. The entire coding region for Ad12 E1B 55-kDa protein was carboxyl terminally tagged with six-His residues by PCR with primers Ad12E1B-Nde (5′-**ACATATG**GAGCGAGAAATCCCACCT-3′, *Nde*I site in boldface) and Ad12E1B-His (5′-**AGAATTC**TCAGTGGTGGTGGTGGTGGTGgatccGTTGTCGTCTTCATCACTTGA-3′, *Eco*RI site in boldface, *Bam*HI site in lowercase, and six-His region underlined). The PCR fragment was cloned into *Nde*I and *Eco*RI sites of pET-22b(+) (Novagen). The coding sequence for the amino-terminal portion up to residue K-158 of Ad12 E1B 55-kDa protein was generated by PCR with primers Ad12E1B-Nde and E1BH3 (5′-AAAA**GCTT**CTTAATAGCACACTCCATATCCTC-3′, *Hin*dIII site in boldface and Ad12 sequence underlined), which was cloned into the *Nde*I and *Hin*dIII sites of pET-22b(+). These constructs were verified by DNA sequencing. These plasmids were introduced into *E. coli* strain BL21 (DE3), and the recombinant proteins were expressed at 30°C upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) for 3 h. The N-terminal fragment of the Ad2 E1B 55-kDa protein (aa 1 to 161) was cloned into pQE30 (Qiagen) and expressed in *E. coli* strain XL1-Blue with IPTG induction.

Protein purification. All proteins were affinity purified with Ni-NTA (nickel-nitrilotriacetic acid) agarose (Qiagen). All purification steps were carried out at 4°C. To purify proteins expressed in Sf9 cells, extracts were prepared from infected cells by one cycle of freeze and thaw in buffer A (20 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 20 mM imidazole, 0.5% Nonidet P-40, 10% glycerol, 10 mM β-mercaptoethanol) supplemented with 1× protease inhibitor cocktail (16 μg of benzamidin HCl per ml, 10 μg of phenanthroline per ml, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 10 μg of pepstatin A per ml, 1 mM phenylmethylsulfonyl fluoride [PMSF]). The extracts were incubated for 30 min with slow rotation. The extracts were then centrifuged and filtered through an 0.22-μm-pore-size filter. The cleared extracts were mixed with Ni-NTA agarose and incubated for 4 h with slow rotation. The resulting agarose was washed three times with buffer A containing 60 mM imidazole, and the proteins were eluted using buffer A containing 200 mM imidazole. Eluted polypeptides were dialyzed in storage buffer (20 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 50% glycerol, 0.1 mM 1,4-dithiothreitol), 0.1 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine) overnight.

To purify proteins expressed in *E. coli*, the bacterial pellets were resuspended in buffer A supplemented with 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, and 0.5 mM PMSF. After sonication and centrifugation, the extracts were filtered through an 0.22-μm-pore-size filter and were incubated with Ni-NTA agarose for 4 h with slow rotation. The other steps in the purification protocol are the same as described above.

Acetyltransferase assay. The human PCAF was purified as described previously (68) and used as acetylase. Purified p53 was subject to acetylation by PCAF in the presence or absence of E1B 55-kDa protein as specified in Fig. 2. Protein samples were incubated at 30°C for 30 to 60 min in a total volume of 20 μl containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM 1,4-dithiothreitol, 1 mM PMSF, 0.1 mM EDTA, 10 mM sodium butyrate, and 90 pmol of 1-^{14}C-acetyl coenzyme A (55 mCi/mmol; Amersham). The reaction mixtures were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were stained with Coomassie brilliant blue, dried, and were subjected to autoradiography.

Detection of in vivo p53 acetylation by PCAF. G401, G401-CC3, and 293 cells were grown for 24 h to 60 to 70% confluence. Cells were washed twice with

phosphate-buffered saline (PBS) and then grown in complete medium supplemented with the deacetylase inhibitor trichostatin A (TSA; Sigma) at a final concentration of 5 μM. At different time points following the addition of TSA, cells were washed twice with ice-cold PBS and lysed on ice in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg of aprotin per ml, 10 μg of leupeptin per ml, 5 μg of pepstatin per ml, 0.5 mM PMSF, and 5 μM TSA). Lysates were clarified at 20,000 × *g* for 30 min at 4°C. Before IP, each sample (containing 2.5 mg of total cellular proteins) was precleared by protein G-agarose (Roche Molecular Biochemicals) for 1 h. Subsequently, 2.5 μg of affinity-purified mouse monoclonal anti-p53 antibody DO-1 (Santa Cruz Biotechnology) was added to the precleared lysates and incubated on ice for 1 h. Then protein G-agarose was added, and the IP mixture was further incubated at 4°C for 1 h with rotation. The beads were collected by centrifugation and then washed five times with ice-cold lysis buffer. The amount of p53 protein in the immunoprecipitates was estimated by Western blotting using goat polyclonal anti-p53 antibody p53 FL-393-G (Santa Cruz Biotechnology) as primary antibody. To determine the acetylation status of p53 in these cells, the same immunoprecipitates with an equal amount of p53 protein were subjected to SDS-PAGE and acetylated p53 was detected with rabbit anti-acetylated p53 (lysine-320) antiserum (Upstate Biotechnology) in Western blot analysis using the enhanced chemiluminescence method (ECL; Amersham-Pharmacia) with an appropriate peroxidase-conjugated secondary antibody.

Electrophoretic mobility shift assay. The DNA-binding activity of p53 in the nuclear extracts of several cell lines was assayed using the NUSHIFT kit (Geneka Biotechnology, Inc.) according to the protocol provided by the manufacturer. The nuclear extracts of G401, G401-CC3, and 293 cells containing approximately equal amounts of p53 were incubated with the oligonucleotide containing consensus p53-binding sites, 5′-AGC**TGGACATGCCCGGGCATGTC**C-3′ (the consensus binding sequence is in bold-face), which was labeled with [γ-^{32}P]ATP. An excessive amount (200-fold over that of labeled probe) of unlabeled WT oligonucleotide or a mutant oligonucleotide, 5′-AGCT**GGATCGCCCCGGGC ATGT CC**-3′ (mutated nucleotides are underlined), was used in the competition assay. The Raji nuclear extract was supplied in the kit and used as positive control. Two microliters of PAb421 anti-p53 monoclonal antibody (100 μg/ml; Calbiochem) was added per assay in some reactions to supershift the p53-DNA complex. The reactions were resolved on a 5% polyacrylamide gel and run at 4°C. The gel was dried and subjected to autoradiography.

IP. In each IP experiment, approximately 0.5 to 1 μg of each purified protein was incubated with an appropriate antibody in NET-gel buffer (20 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 0.5% Nonidet P-40, 10% glycerol, and 5 mM EDTA) for at least 1 h with rotation at 4°C. The amount of antibody used per IP assay is different depending on specific antibodies; the typical amount is 100 μl of hybridoma supernatant (2A6; anti-Ad2 E1B 55-kDa protein), 3 μl of purified monoclonal antibody against p53 (DO-1 [Santa Cruz Biotechnology] or PAb421 [Calbiochem]), 6 μl of anti-FLAG M2 antibody (Sigma), or 3 μl of rabbit antiserum against the N terminus of Ad12 E1B 55-kDa protein. Protein A-agarose beads (15 μl; Roche Molecular Biochemicals) were added into the protein-antibody mixture and incubated at 4°C for 1 h with rotation. The beads were collected by centrifugation and washed three times. The first wash was done with NET-gel buffer supplemented with NaCl to a final concentration of 0.5 M; the second wash was with NET-gel buffer supplemented with 0.1% SDS, and the third wash was carried out with 10 mM Tris-HCl (pH 7.4) with 0.1% Nonidet P-40. The beads were pelleted by centrifugation and mixed with 30 μl of 1× SDS loading buffer. The precipitated proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane in 25 mM Tris base–190 mM glycine at 50 V for 3 h at 4°C. The coprecipitated proteins were detected using an appropriate antibody with the enhanced chemiluminescence (ECL) kit.

To detect E1B-PCAF interaction in vivo, 293 cells were washed twice with PBS and lysed on ice with ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5). Lysates were clarified by centrifugation and then precleared with protein G-agarose. The IP antibody was incubated with the cell lysates at 4°C for 1 h. Protein G-agarose was added and incubated at 4°C for 1 h. The beads were collected by centrifugation and then washed five times with ice-cold RIPA buffer. For detecting Ad12 E1B-PCAF interaction, G401-CC3 cell lysates were subjected to IP using anti-E1B, and the immunoprecipitates were analyzed by Western blotting using anti-PCAF. In addition, G401-CC3 cells were transfected with pCX-Flag-PCAF by lipofection. Forty-eight hours after transfection, cell lysates were made and then subjected to IP as described above for 293 cells.

Yeast two-hybrid assay. Various DNA fragments spanning different regions of the Ad2 or Ad12 E1B 55-kDa protein open reading frame were fused either to yeast GAL4 activation domain (AD) in plasmids pGAD-C(x) or to GAL4 DNA-binding domain (BD) in plasmids pGBDU-C(x) (22). Similarly, a DNA fragment encoding the full-length human PCAF was cloned into pGAD-C3 and pGBDU-C3 (22). A series of plasmids containing varying N-terminal deletions of human p53 cDNA that were fused to GAL4 AD were described previously (21) and were kindly provided by Stanley Fields. The p53 AD (aa 1 to 145) and sequence-specific BD (aa 76 to 315) were separately cloned into pGAD-C1 and pGBDU-C1.

The yeast strain PJ69-4A (*MATa trp1-190 leu2-3,112 ufa3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) was used for all two-hybrid assays as described previously (22). Plasmids are introduced into PJ69-4A by the standard lithium acetate transformation method. To test potential protein-protein interaction, transformants were screened for growth in medium lacking histidine but in the presence of 5 mM 3-aminotriazol (3-AT) (His$^+$ phenotype) or lacking adenine (Ade$^+$ phenotype) or assayed for β-galactosidase activity (blue phenotype) in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or by quantitative measurement using Galacto-Light reagents (Tropix) and a luminometer.

Reverse two-hybrid assay. The *Bam*HI-*Bgl*II fragment encoding Ad2 E1B 55-kDa protein aa 1 to 437 (missing 58 aa residues in the C terminus) from pGEM-T/Ad2 HA-E1B and the *Eco*RI fragment encoding the full-length Ad12 E1B from pGEM-T/Ad12 HA-E1B were cloned into plasmid pCu424, which has a copper-inducible promoter and the *Trp1*-selectable marker (28). These plasmids were separately introduced into yeast strain PJ69-4A together with plasmids pGBDU-PCAF and pGAD-p53. Yeast minimal medium lacking copper was supplemented with ascorbic acid (1 mM) and bathocuproinedisulfonate (33 μM). Different amounts of $CuSO_4$ were added for induction of gene expression from pCu424 constructs.

Preparation of yeast cell extracts. Fifty milliliters of overnight culture in selective dropout medium with an optical density at 600 nm (OD_{600}) of between 0.4 and 0.5 was centrifuged, and the pellet was resuspended in 100 μl of cold trichloroacetic acid (TCA) buffer (20 mM Tris HCl [pH 8.0], 50 mM ammonium acetate, 2 mM EDTA, 1 mM PMSF, 1× protease inhibitor solution containing 0.1 μg of pepstatin A per ml, 0.03 μM leupeptin, 145 μM benzamidine, and 0.37 μM aprotinin) per 7.5 OD_{600} units (OD_{600} units = volume [ml] × OD_{600} [1 ml]). An equal volume of glass beads and 20% TCA were added; the mixture was vortexed for 1 min with a 30-s pause on ice, and the vortexing was repeated five times. The suspension of broken cells was transferred into another tube, and the glass beads were washed with 250 μl each of TCA and 20% TCA. The combined broken-cell suspension was centrifuged, and the pellet was resuspended in 10 μl of TCA-Laemmli loading buffer (3.5% [wt/vol] SDS, 14% [vol/vol] glycerol, 120 mM Tris base, 8 mM EDTA, 0.01% bromophenol blue, 0.7 M β-mercaptoethanol, 2 mM PMSF, 1× protease inhibitor solution) per OD_{600} unit. The samples were heated at 100°C for 10 min and centrifuged. The supernatant was subjected to SDS-PAGE and Western blot analysis to detect proteins under study.

RESULTS

Expression and purification of E1B 55-kDa protein and p53. Recombinant baculoviruses expressing Ad2, Ad12 E1B 55-kDa protein, or p53 were constructed using the Bac-to-Bac baculovirus expression system (Gibco-BRL). We found that the levels of protein expression were highest at 72 h postinfection for Ad2 and Ad12 E1B 55-kDa protein as well as for p53. We also found that the level of p53 expression was significantly higher than that of either Ad2 or Ad12 E1B 55-kDa protein. The reason for this is unknown but could stem from hindrance of Sf9 cell growth by the 55-kDa oncoprotein, as it also slows the growth of human cells (data not shown).

The Ad12 E1B 55-kDa protein and its amino-terminal portion (Ad12 E1B-N, aa 1 to 158) were also expressed in *E. coli*. The expression level of WT Ad12 E1B 55-kDa protein was very low at 37°C, due to severe degradation, whereas Ad12 E1B-N was expressed at high levels but remained largely insoluble in the inclusion body at 37°C. Thus, we shifted bacterial growth temperature to 30°C for their expression.

Since all the recombinant proteins were tagged with six His residues, they were affinity purified using Ni-NTA agarose. Figure 1 shows Coomassie brilliant blue-stained SDS-polyacrylamide gels of purified proteins. Both Ad2 and Ad12 E1B 55-kDa proteins migrate in parallel with glutamic dehydrogenase (molecular mass of 55.6 kDa) in the protein marker lane, whereas the p53 carrying both the six-His and FLAG tags migrates slightly faster than this marker protein. The identity of these proteins was confirmed by Western blotting using antibodies 2A6 (48), rabbit polyclonal antiserum against Ad12 E1B 55-kDa protein (32), and anti-p53 antibody DO-1 (data not shown).

E1B 55-kDa protein specifically inhibits p53 acetylation by PCAF in vitro. p53 is covalently modified by phosphorylation and acetylation, and such modifications have profound effects on p53 functions (42). p53 is acetylated by transcriptional coactivator p300 at Lys-373 and Lys-382 and by PCAF at Lys-320.

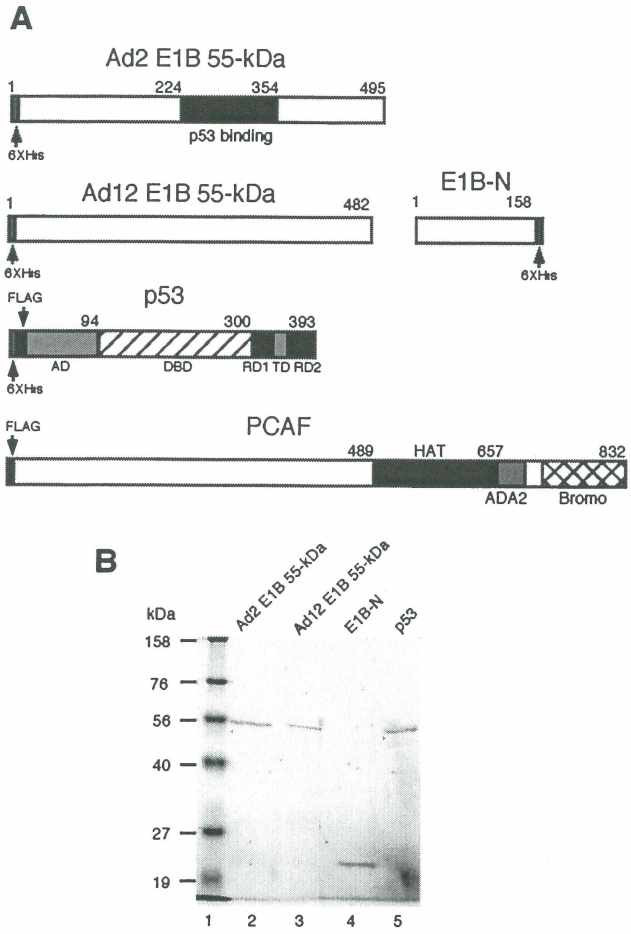

FIG. 1. Purification of E1B 55-kDa protein, E1B-N, and p53. (A) Schematic drawings of proteins used in this study. His or FLAG tag was attached either at the N terminus or at the C terminus of a protein as indicated. Known functional domains of these proteins are denoted: DBD, sequence-specific BD; RD, regulatory domain; TD, tetramerization domain; HAT, HAT domain; ADA2, alteration and/or deficiency in activation (4, 63); Bromo, bromodomain (8). (B) SDS-PAGE analysis of purified proteins. Full-length Ad2, Ad12 E1B 55-kDa, and p53 proteins were expressed in insect Sf9 cells, and the N terminus of Ad12 E1B (E1B-N) was expressed in *E. coli*. The proteins were purified using Ni-NTA agarose, an aliquot of each protein was analyzed by SDS–10% PAGE, and the gel was stained with Coomassie brilliant blue. The protein identity is indicated on top of each lane.

Importantly, acetylation of p53 at these sites enhances sequence-specific DNA-binding activity of p53 (17, 35, 47), thereby activating p53. As Ad E1B 55-kDa oncoprotein represses p53-mediated transcriptional transactivation (55, 69), we reasoned that E1B might do so by inhibiting covalent modifications of p53. To test this possibility, we carried out acetylation assays using PCAF as the acetylase and p53 as the substrate in the absence or presence of E1B 55-kDa protein. The effects of E1B 55-kDa proteins on PCAF acetylation are presented in Fig. 2. Consistent with previous reports, PCAF acetylates itself and p53 (Fig. 2A, lanes 1 and 2) (47, 68). The presence of either purified Ad2 E1B 55-kDa protein (lane 3), Ad12 E1B 55-kDa protein (lane 4), or Ad12 E1B N-terminal domain (E1B-N) (lane 5) resulted in striking (70 to 95%) reduction of p53 acetylation, whereas PCAF autoacetylation remained largely unaffected by E1B (lanes 3 to 5 and 7 to 9). PCAF does not acetylate WT E1B 55-kDa protein (lanes 7 and 8) or E1B-N (lane 9) (the acetylated species that ran at the dye front in lanes 5 and 9 containing E1B-N are unknown). As a positive control, histones were used as substrate for PCAF acetylation (lane 10). The inhibition of p53 acetylation by E1B was concentration dependent. As shown in Fig. 2B, the addition of increasing amounts of E1B-N gradually inhibited p53 acetylation by PCAF. At high E1B-N concentrations, p53 acetylation was hardly detectable (Fig. 2B, lane 5). Similar dosage-dependent inhibition was also observed with WT E1B 55-kDa protein (Fig. 2C). The observed inhibition of p53 acetylation by PCAF is unlikely to be due to a contaminant in the E1B preparation since full-length E1B protein was purified from Sf9 cells and E1B-N was purified from *E. coli*. Furthermore, the purified N-terminal fragment of Ad2 E1B 55-kDa protein (aa 1 to 161) did not inhibit acetylation catalyzed by PCAF (Fig. 2D, lanes 3 and 4). (Note that this protein fragment did not stain well with Coomassie blue. We loaded 0.17 μg [~8 pmol] in Fig. 2D, lane 3, and 1.2 μg in lane 4 [~60 pmol]). This Ad2 E1B fragment shares little sequence identity with the Ad12 E1B N-terminal fragment (E1B-N) and does not bind to either p53 or PCAF (see below). In additional control experiments, high concentrations of bovine serum albumin (BSA) did not inhibit p53 acetylation by PCAF or its autoacetylation (Fig. 2E, lanes 2 and 3).

To investigate whether E1B 55-kDa protein inhibits histone acetylation by PCAF, purified Ad2 and Ad12 E1B 55-kDa proteins as well as E1B-N (Fig. 2F, lanes 2 to 4) were incubated with histones and PCAF. Acetylation of neither core histones nor histone H1 was inhibited by WT E1B (lanes 2 and 3) or E1B-N (lane 4), although p53 acetylation was severely inhibited or completely abolished at the E1B concentrations used in the assays (Fig. 2A). We conclude that E1B 55-kDa protein does not affect PCAF histone acetylase (HAT) activity. Curiously, acetylation of histone H1 was dramatically increased in the presence of a high concentration of E1B-N (Fig. 2F, lane 4). The reason for this is unknown.

Acetylation of p53 is suppressed in vivo. We then tested if E1B 55-kDa oncoprotein would affect acetylation of p53 by PCAF in vivo. We first quantified the total amount of p53 protein in cell lysates with an IP-Western blot protocol using mouse monoclonal anti-p53 antibody DO-1 for IP and goat polyclonal antibody anti-full-length p53 for Western blot analysis. The results are shown in Fig. 3A. The same immunoprecipitates containing approximately the same amounts of p53 were then subjected to SDS-PAGE and Western blot analysis, and acetylated p53 was detected with rabbit polyclonal antiserum against p53 acetylated at Lys-320, the specific acetylation site of PCAF (35, 47). As shown in Fig. 3B, acetylated p53 was detectable in G401 cells at 1.5 h and accumulated gradually at 3 and 9 h after addition of deacetylase inhibitor TSA (lanes 4, 7, and 10). In contrast, acetylated p53 was not detectable or was dramatically reduced in G401-CC3 cells that express Ad12 E1B 55-kDa protein and 293 cells that express Ad5 E1B 55-kDa protein (Fig. 3B, lanes 5, 6, 8, 9, 11, and 12), although the same amount of total p53 was present in each sample (Fig. 3A). The specificity of anti-acetyl-p53 antibody was confirmed, as it was reactive only to purified p53 that was acetylated by PCAF in vitro (lane 1), but not to the same amount of p53 that was not treated with PCAF (lane 3). Thus, p53 acetylation was severely suppressed by the E1B 55-kDa protein in vivo.

The sequence-specific DNA binding of p53 is inhibited in G401-CC3 and 293 cells. Since acetylation of p53 by p300 and PCAF stimulates sequence-specific DNA-binding activity of p53 (17, 35, 47), and we showed above that E1B 55-kDa protein inhibits acetylation of p53, one would expect that p53 from cells expressing E1B may be less competent to bind to its

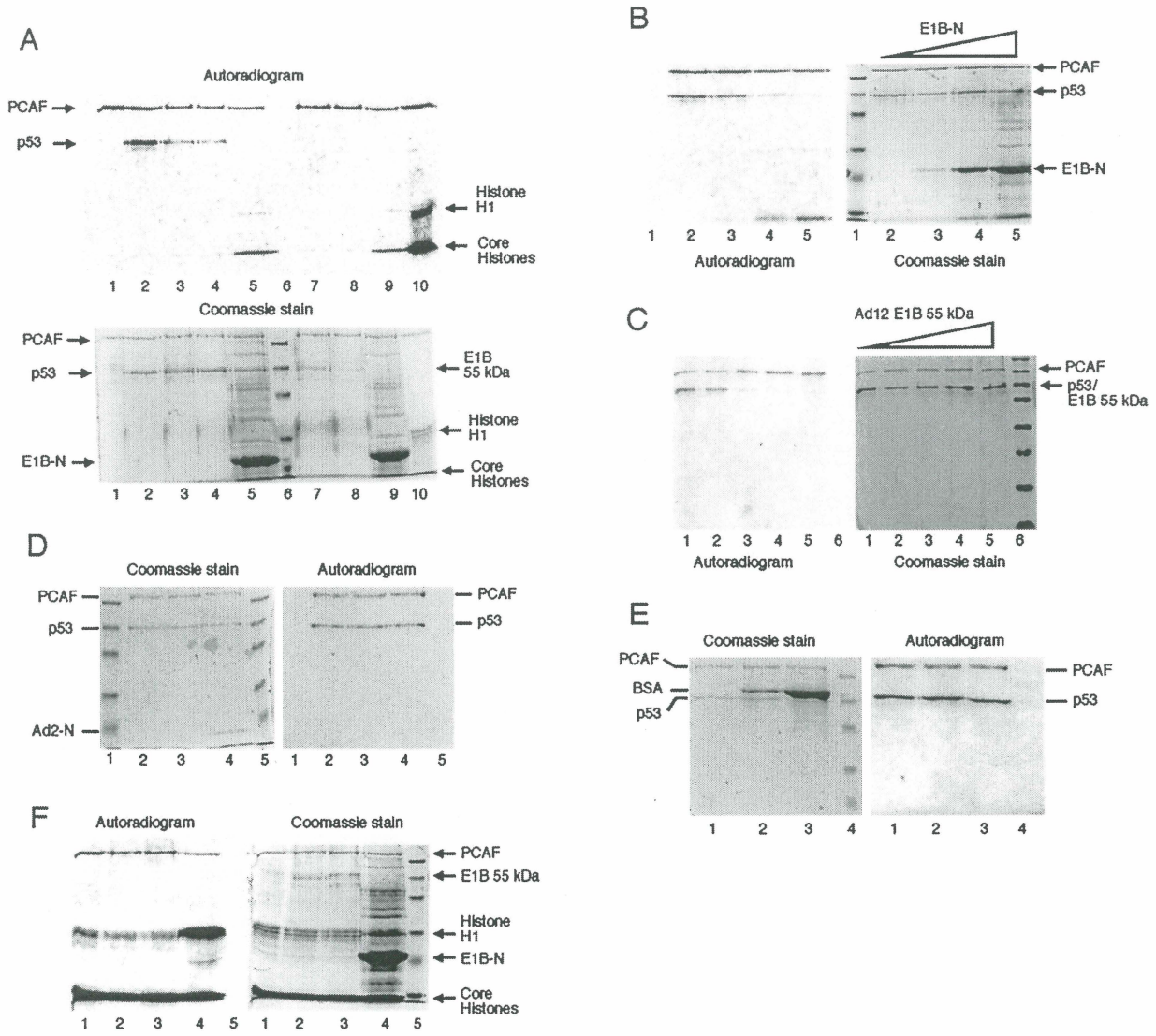

FIG. 2. E1B 55-kDa protein specifically inhibits acetylation of p53 by PCAF. (A) E1B 55-kDa protein inhibits acetylation of p53 by PCAF but not its autoacetylation. PCAF (4.3 pmol) was incubated alone (lane 1) or with 5.4 pmol of p53 (lanes 2 to 5) in the absence (lanes 1 and 2) or presence (lanes 3 to 5) of E1B (Ad2 E1B 55-kDa protein [3.5 pmol, lane 3], Ad12 E1B 55-kDa protein [3.5 pmol, lane 4], and E1B-N [60 pmol, lane 5]). Lane 6 is molecular weight markers. The same amount of PCAF (4.3 pmol) was incubated with full-length E1B 55-kDa protein (Ad2 [3.5 pmol, lane 7]) and Ad12 [3.5 pmol, lane 8]) and E1B-N (60 pmol, lane 9) in the absence of p53. Lane 10 shows acetylation of histones (2.0 μg; Sigma) by PCAF. The top portion is an autoradiogram of the Coomassie blue-stained gel (10%, bottom portion). (B) Inhibition of p53 acetylation by PCAF with increasing concentrations of E1B-N. PCAF (4.3 pmol) was incubated with p53 (5.4 pmol, lane 2) or with p53 plus increasing amounts of E1B-N at 6.0 pmol (lane 3), 30 pmol (lane 4), and 60 pmol (lane 5). The left part is an autoradiogram of the Coomassie blue-stained gel (10%) shown on the right. (C) Inhibition of p53 acetylation by PCAF with increasing concentrations of Ad12 E1B. PCAF (4.3 pmol) was incubated with p53 (5.4 pmol, lane 1) or with p53 plus increasing amounts of Ad12 E1B 55-kDa protein at 1.0, 4.0, 6.0, and 8.0 pmol (lanes 2 to 5, respectively). The left part is an autoradiogram of the Coomassie blue-stained gel (10%) shown on the right. (D) The N-terminal domain of Ad2 E1B 55-kDa protein does not inhibit acetylation of p53 by PCAF. PCAF (4.3 pmol) was incubated with p53 (5.4 pmol, lanes 2 to 4) in the absence (lane 2) or presence (lanes 3 and 4) of Ad2 E1B N-terminal domain (aa 1 to 161) (8 pmol [lane 3] and 60 pmol [lane 4]). Lanes 1 and 5 are molecular weight markers. The Coomassie blue-stained gel (10%) and its autoradiogram are shown on the left and right, respectively. (E) BSA does not affect acetylation of p53 by PCAF. PCAF (4.3 pmol) was incubated with p53 (5.4 pmol, lanes 1 to 3) in the absence (lane 1) or presence (lanes 2 and 3) of BSA (8 pmol [lane 2] and 60 pmol [lane 3]). Lane 4 is molecular weight markers. The Coomassie blue-stained gel (10%) and its autoradiogram are shown on the left and right, respectively. (F) E1B does not affect acetylation of histones by PCAF. Histones (2.0 μg) were subjected to acetylation by PCAF (4.3 pmol) in the absence of E1B (lane 1) or the presence of 3.5 pmol of Ad2 E1B 55-kDa protein (lane 2), 3.5 pmol of Ad12 E1B 55-kDa protein (lane 3), or 300 pmol of E1B-N (lane 4). Lane 5 is molecular weight markers. At left is an autoradiogram, and at right is the Coomassie blue stain of the same gel (10%).

cognate DNA sequence. To test this possibility, we monitored the sequence-specific DNA-binding activity of p53 in nuclear extracts of G401, G401-CC3, and 293 cells in an electrophoretic mobility shift assay using a radiolabeled oligonucleotide bearing consensus p53-binding sites (oligonucleotide WT in Fig. 4). As shown in Fig. 4, two specific bands denoted with a hollow arrowhead, presumably p53-DNA complexes, were readily detectable in G401 nuclear extracts (lanes 3 and 4). These two bands disappeared almost completely in the presence of competing unlabeled oligonucleotide WT (lane 5). However, these two bands were not detectable in the nuclear extracts of G401-CC3 (lane 7) and 293 (lane 11) cells, although these nuclear extracts contained about the same amount of p53 protein as that of G401 (Fig. 4A, top). As expected, monoclonal antibody PAb421, which recognizes an epitope in p53 RD2 (aa 371 to 381), enhances DNA binding by p53 (lanes 2 and 4;

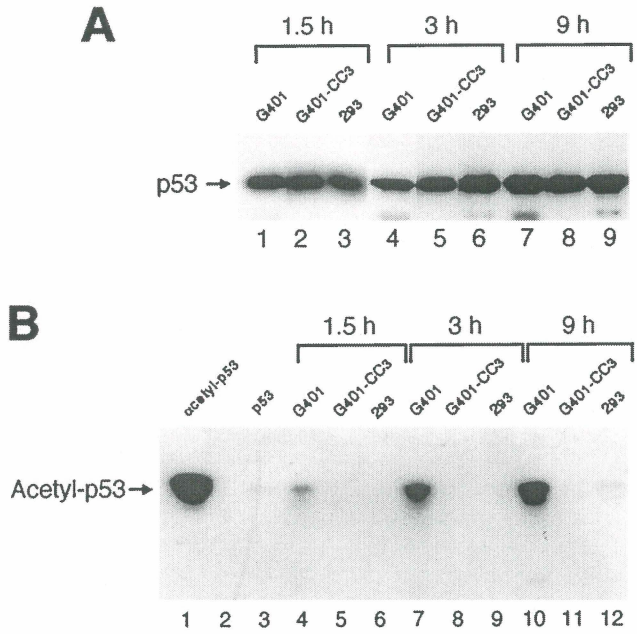

FIG. 3. E1B 55-kDa proteins inhibit acetylation of p53 in vivo. (A) Quantification of total p53 protein. G401 cells do not express E1B 55-kDa protein, while G401-CC3 expresses Ad12 E1B 55-kDa protein and 293 produces both Ad5 E1A and E1B proteins. Cells were harvested at 1.5 h (lanes 1 to 3), 3 h (lanes 4 to 6), or 9 h (lanes 7 to 9) after addition of deacetylase inhibitor TSA (5 μM), and cell extracts were subjected to IP with mouse monoclonal antibody DO-1 and Western blot analysis with goat anti-p53 polyclonal antibody. (B) Detection of acetylated p53. The same immunoprecipitates containing approximately equal amounts of total p53 protein as shown in panel A were resolved by SDS-PAGE, and the acetylated fraction of p53 was detected by Western blot analysis with an anti-acetyl-p53 (Lys-320) antibody (Upstate Biotechnology). Lanes 1 and 3 show p53 (9 pmol) acetylated by PCAF in vitro and nontreated, respectively. Lane 2 is an empty lane.

the supershifted complex is denoted with a solid arrowhead). PAb421 was able to stimulate p53's DNA-binding activity in G401-CC3 nuclear extracts (lanes 8 and 10). Surprisingly, it had virtually no effect on p53's DNA-binding activity in 293 nuclear extracts (lanes 12 and 14). As both G401-CC3 and 293 express E1B 55-kDa protein, these results indicate that reduced acetylation of p53 in the presence of E1B impairs p53's sequence-specific DNA-binding activity. As binding of p53 to its cognate DNA sequence is required for its transactivation function, inhibition of p53 acetylation by E1B may result in diminished transactivation by p53. Indeed, the level of p21 protein, whose expression is stimulated by p53, was significantly lower in G401-CC3 and 293 cells than in G401 cells in the presence of approximately equal amounts of p53 (Fig. 4B).

PCAF binds directly to E1B 55-kDa protein and p53. To understand the mechanisms by which E1B inhibits p53 acetylation by PCAF, we studied possible interactions among PCAF, p53, and E1B by IP and yeast two-hybrid assays. Figure 5A shows IP results with the rabbit polyclonal antibody anti-E1B against Ad12 E1B 55-kDa protein and mouse monoclonal antibody DO-1 against p53. The precipitates were subjected to Western blot analysis using mouse monoclonal antibody M2 against the FLAG tag. Purified p53 and PCAF were both tagged with FLAG at the amino terminus and loaded directly on the gel without IP (lanes 1 and 2; about 10% of the amount used for binding reactions). The small species in these two lanes are likely due to partial degradation of p53 (lane 1) and PCAF (lane 2). The polyclonal antibody anti-E1B precipitates PCAF in the presence of Ad12 E1B (lane 6), but this antibody

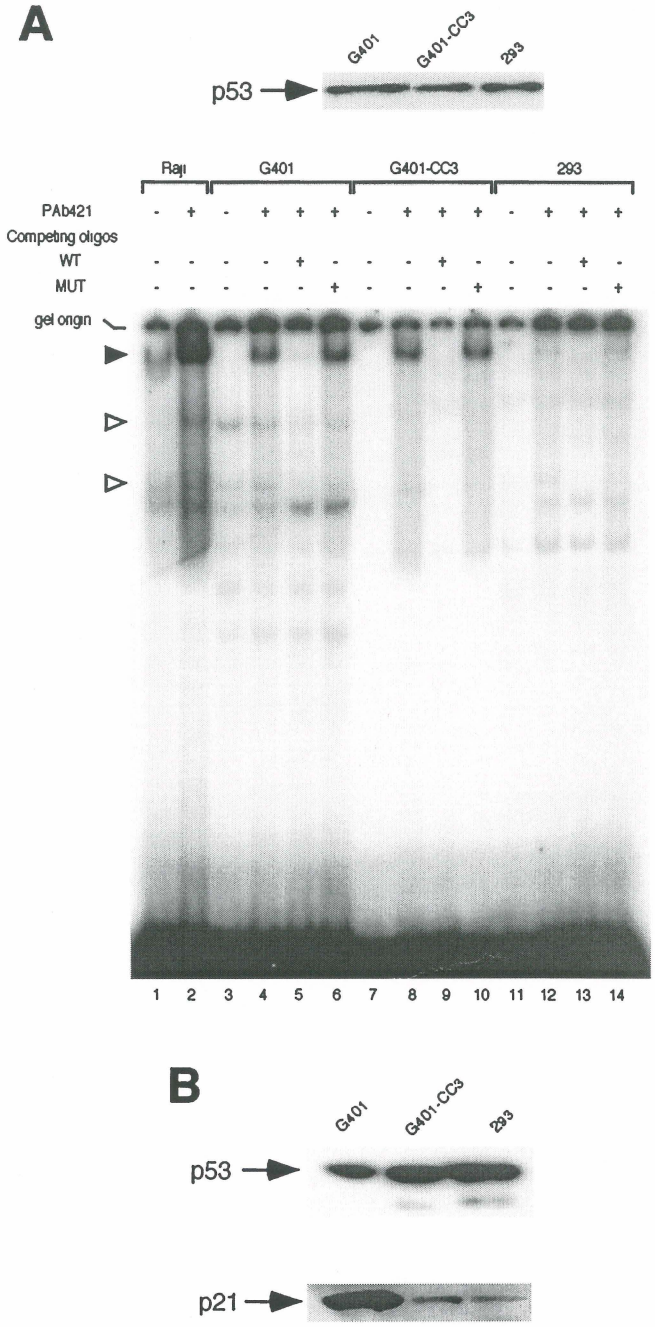

FIG. 4. p53 sequence-specific DNA-binding activity in cells expressing E1B 55-kDa protein. (A) Electrophoretic mobility shift assay of p53 DNA-binding activity. The nuclear extracts of cell lines G401, G401-CC3, and 293, which contained about equal amounts of p53 as shown on the top of panel A, were incubated with a radioactive oligonucleotide bearing consensus p53-binding sites (WT). Competing unlabeled WT oligonucleotide or mutant oligonucleotide (MUT) as well as monoclonal anti-p53 antibody PAb421 was added in some reactions as indicated. Raji nuclear extract was used as positive control. Specific p53-DNA complexes are denoted with arrowheads. (B) p21 levels in G401, G401-CC3, and 293 cells. The p53 levels in the total cell extracts of these three cell lines were estimated by Western blotting (top), and the p21 protein concentration in total cell extracts containing about equal amounts of p53 was determined by Western blotting using an anti-p21 polyclonal antibody (C-19; Santa Cruz Biotechnology).

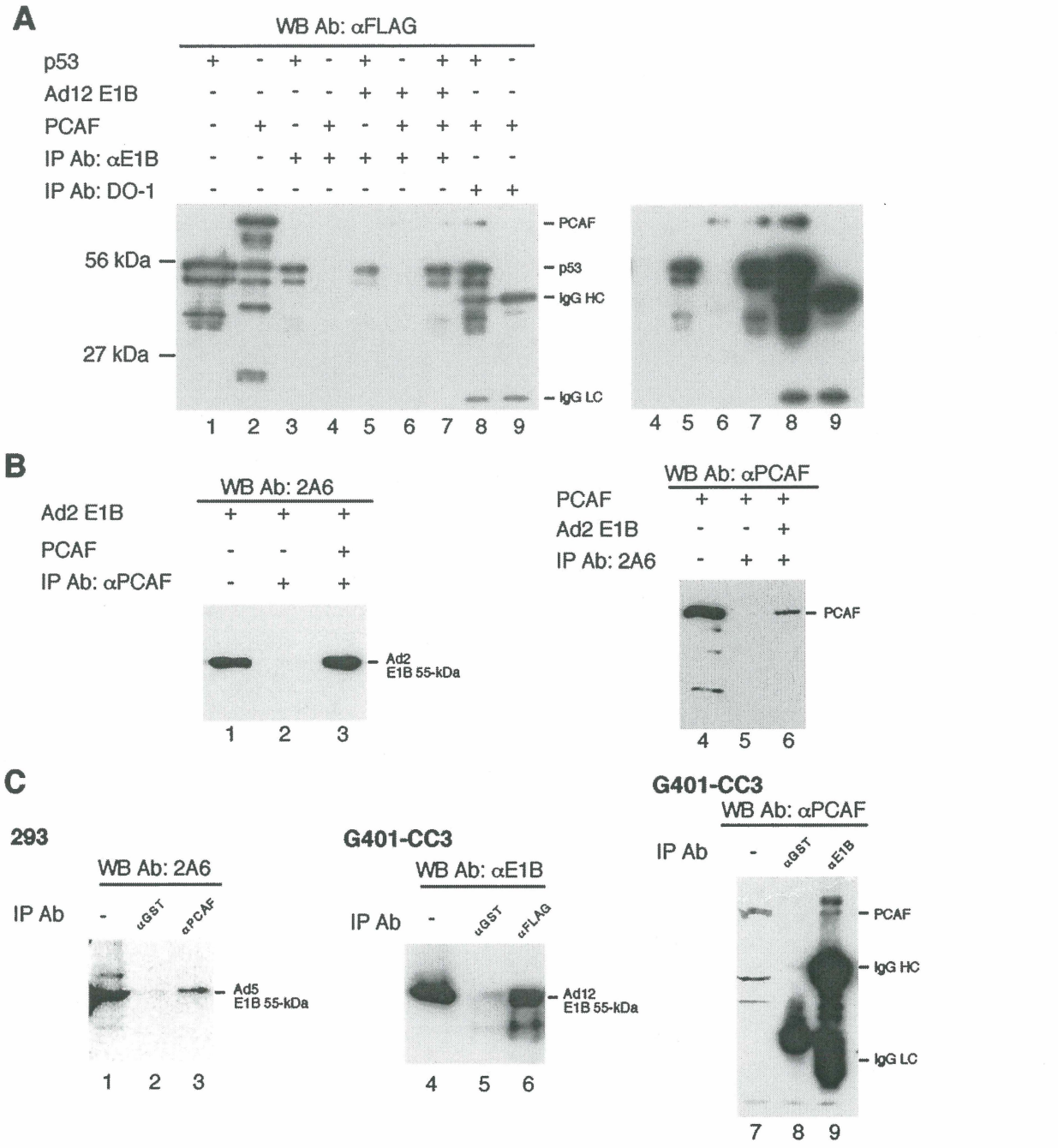

FIG. 5. Interactions among p53, E1B, and PCAF. For in vitro assay, purified p53 (5.4 pmol), E1B 55-kDa protein (7 pmol), and PCAF (6 pmol) were incubated and subjected to IP with different antibodies indicated on the top of each panel. The precipitates were subjected to Western blot analysis with indicated antibodies. The abbreviations IgG HC and IgG LC denote IgG heavy and light chains, respectively. (A) Ad12 E1B 55-kDa protein binds directly to PCAF. About 10% of the amount of both p53 (lane 1) and PCAF (lane 2) used for the binding reactions was loaded directly on the gel without IP. p53 (lane 3) or PCAF (lane 4) was incubated with rabbit antiserum raised against Ad12 E1B 55-kDa protein (anti-E1B) and subjected to IP. IP of p53 and Ad12 E1B 55-kDa protein (lane 5); PCAF and Ad12 E1B 55-kDa protein (lane 6); and PCAF, p53, and Ad12 E1B 55-kDa protein (lane 7) was done using anti-E1B. Monoclonal antibody DO-1 against p53 was incubated with PCAF and p53 (lane 8) or PCAF alone (lane 9) and subjected to IP. The immunoprecipitates were detected with mouse monoclonal antibody M2 against FLAG tag (anti-FLAG). For a clear visualization of the PCAF band, a longer exposure of the same blot is shown for the right portion (from lanes 4 to 9) of the image. (B) Ad2 E1B binds to PCAF. About 10% of the amount of both Ad2 E1B (lane 1) and PCAF (lane 4) used for the binding reactions was loaded directly on the gel without IP. Rabbit polyclonal antibody against PCAF (anti-PCAF) was incubated with Ad2 E1B (lane 2) or Ad2 E1B plus PCAF (lane 3) and subjected to IP. The immunoprecipitates were probed with mouse monoclonal anti-E1B antibody 2A6 in a Western blot analysis. Similarly, 2A6 was incubated with PCAF (lane 5) or Ad2 E1B plus PCAF (lane 6) and subjected to IP. PCAF in the immunoprecipitates was detected with anti-PCAF. (C) E1B 55-kDa proteins bind to PCAF in vivo. Cell lysate of 293 cells was subjected to IP with anti-PCAF (lane 3) or anti-GST (lane 2). The precipitated E1B 55-kDa protein was detected by 2A6. Lane 1 shows a direct load of 293 cell lysate (10% of that used for IP). To detect interaction between PCAF and Ad12 E1B 55-kDa protein, plasmid pCX-Flag-PCAF was transfected into G401-CC3 cells which constitutively express Ad12 E1B 55-kDa protein. The lysate of transfected cells was subjected to IP with anti-FLAG (lane 6) or anti-GST (lane 5), and the precipitates were analyzed in a Western blot assay using anti-E1B. Lane 4 is a direct load of G401-CC3 cell lysate (10% of that used for IP). In reciprocal experiments, G401-CC3 cell lysate was subjected to IP with anti-E1B (lane 9) or anti-GST (lane 8), and the precipitates were subjected to a Western blot analysis using anti-PCAF. Lane 7 is a direct load of G401-CC3 cell lysate (10% of that used for IP). WB, Western blot; Ab, antibody.

does not directly precipitate PCAF (lane 4). M2 (anti-FLAG) did not recognize any protein species in the preparation of Ad12 E1B 55-kDa protein (data not shown), thereby excluding the possibility that the band seen in lanes 6 and 7 was due to cross-reaction of M2 with Ad12 E1B preparation. Thus, Ad12 E1B 55-kDa protein binds directly to PCAF. The simultaneous presence of PCAF, p53, and Ad12 E1B did not affect PCAF precipitation by anti-E1B (lane 7). Similarly, DO-1 precipitates PCAF along with p53 (lane 8) but does not precipitate PCAF directly (lane 9). A longer exposure of the blot allowed clear visualization of PCAF (see lanes 6 to 8 in the right panel). Therefore, PCAF also binds directly to p53. We also tested if p53 binds to Ad12 E1B 55-kDa protein. p53 was clearly precipitated by anti-E1B (lane 5), but this antibody can recognize p53 directly (lane 3). Thus, whether Ad12 E1B 55-kDa protein binds directly to p53 remains inconclusive based on these IP results. However, we showed that the two proteins colocalize in the cell (see below).

The interactions between Ad2 55-kDa protein and PCAF were studied by IP using rabbit polyclonal antibody against PCAF (anti-PCAF) as IP antibody and monoclonal antibody 2A6 against Ad2 55-kDa protein for Western blot analysis (Fig. 5B). The purified Ad2 E1B 55-kDa protein was loaded directly on the gel (lane 1, 10% of the amount used for binding reactions) as a positive control. Anti-PCAF did not precipitate Ad2 E1B directly (lane 2), but it can recover Ad2 E1B in the presence of PCAF (lane 3). In the reciprocal IP experiments using antibody 2A6, PCAF coprecipitated with Ad2 E1B (lane 6), but not directly by 2A6 (lane 5).

To determine PCAF-E1B interaction in vivo, anti-PCAF was again used as IP antibody for human 293 cell lysates. As shown in Fig. 5C, Ad5 E1B 55-kDa protein was precipitated by anti-PCAF (lane 3), but not by irrelevant antibody anti-glutathione *S*-transferase (anti-GST) (lane 2). Lane 1 is a direct load of 293 cell lysates. To detect interactions between PCAF and Ad12 E1B 55-kDa protein we chose to transfect G401-CC3 cells with plasmid pCX-Flag-PCAF (68) just to avoid the visualization of immunoglobulin heavy and light chains because both anti-PCAF and anti-E1B were raised in rabbits. The transfected cell lysates were subjected to IP with mouse monoclonal antibody M2 against the FLAG tag and anti-GST, and the immunoprecipitates were detected with anti-E1B (rabbit polyclonal antiserum). As shown in Fig. 5C, Ad12 E1B 55-kDa protein was precipitated by M2 (anti-FLAG, lane 6), but not by anti-GST (lane 5). Lane 4 is a direct load of G401-CC3 cell lysates. Furthermore, PCAF was present in the immunoprecipitates using anti-E1B (lane 9), but not in that using anti-GST (lane 8). Lane 7 is a direct load of G401-CC3 cell lysates. Therefore, both Ad5 and Ad12 E1B 55-kDa proteins interact with PCAF in vivo.

Ad12 E1B 55-kDa protein and p53 colocalize in the cell. Whether Ad12 E1B 55-kDa protein and p53 interact with each other is less clear, as inconsistent results were reported in the literature (12, 16, 55, 61, 73). To clarify this issue, we tested whether p53 and Ad12 E1B 55-kDa protein colocalize in cells. G401-CC3 cells, which constitutively express Ad12 E1B 55-kDa protein, were grown on a glass coverslip, fixed, and then stained with anti-p53 antibody DO-1 and anti-Ad12 E1B antibody anti-E1B. p53 was visualized with fluorescein-conjugated anti-mouse immunoglobulin G (IgG) antibody (Fig. 6A), and Ad12 E1B was visualized with Texas red-conjugated anti-rabbit IgG antibody (Fig. 6B). Both p53 and Ad12 E1B 55-kDa protein localize primarily to the nucleus, and the colocalization of the two proteins in the nucleus is quite apparent in the merged image (Fig. 6D). Furthermore, the two proteins colocalize in a dense cytoplasmic body (denoted with arrowheads in Fig. 6A, B, and D). The staining pattern of anti-E1B shown in Fig. 6B was seen only in cells expressing Ad12 E1B 55-kDa protein, as we showed previously (32), excluding the possibility that the pattern was due to cross-reaction of cellular proteins with anti-E1B. Collectively, these results indicate that Ad12 E1B 55-kDa protein also interacts with p53, consistent with recent studies (12, 16, 67).

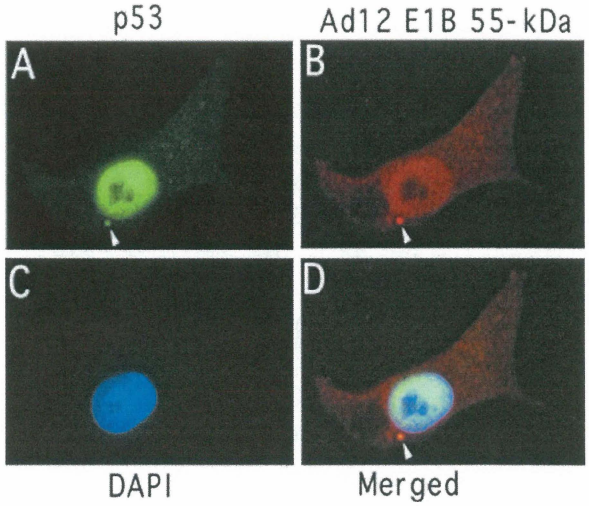

FIG. 6. Subcellular colocalization of Ad12 E1B 55-kDa protein and p53. G401-CC3 cells that constitutively express Ad12 E1B 55-kDa protein were grown on glass coverslip and fixed. Antibody staining of the cells was done as described previously (32). (A) p53 was visualized with anti-p53 antibody DO-1 and fluorescein-conjugated anti-mouse IgG antibody. (B) The Ad12 E1B 55-kDa protein was stained with anti-Ad12 E1B antibody anti-E1B and visualized with Texas red-conjugated anti-rabbit IgG antibody. (C) Nuclear staining with 4′,6′-diamidino-2-phenylindole (DAPI). (D) Merged image of panels A, B, and C. Colocalization of p53 and Ad12 E1B 55-kDa protein in a cytoplasmic body is indicated by an arrowhead.

PCAF binds to the C-terminal domain of p53. We employed the yeast two-hybrid assay to assess which domain of p53 binds to PCAF. The PCAF-GAL4 BD hybrid interacts with p53-GAL4 AD hybrid, resulting in a His^+ phenotype (Fig. 7A), as well as expression of β-galactosidase (data not shown). Progressive deletion of the N terminus of p53 until aa 253 did not attenuate PCAF-p53 interaction (Fig. 7A, sectors 1 to 5). The p53 construct spanning residues 293 and 393 can still bind to PCAF (sector 6), although this construct appears to bind to PCAF with reduced affinity based on β-galactosidase activity (data not shown). The deletion mutant carrying only residues 331 to 393 no longer bound to PCAF (sector 7), and neither did p53 mutants having only the N-terminal 145 aa (sector 8) or the DBD (aa 76 to 315, sector 9). All the p53 constructs were expressed in yeast as judged by Western blot analysis (data not shown), as was the PCAF-GAL4 BD hybrid (see Fig. 9C). Figure 7E summarizes the results of assays for PCAF-p53 interaction. Since PCAF acetylates Lys-320 of p53, these results suggest that a direct physical interaction may be necessary for acetylation. The primary sequence and/or structure of the substrate may determine the specificity of PCAF acetylation. Our results are in agreement with biochemical data reported recently (35).

We also tested interaction between E1B 55-kDa protein and p53 as well as between E1B and PCAF using the two-hybrid assay. We were unable to make the GAL4 BD or AD fusions with the full-length Ad2 E1B gene; these constructs were unstable, as reported previously (9, 32). Nonetheless, several constructs containing a partial Ad2 55-kDa protein sequence were

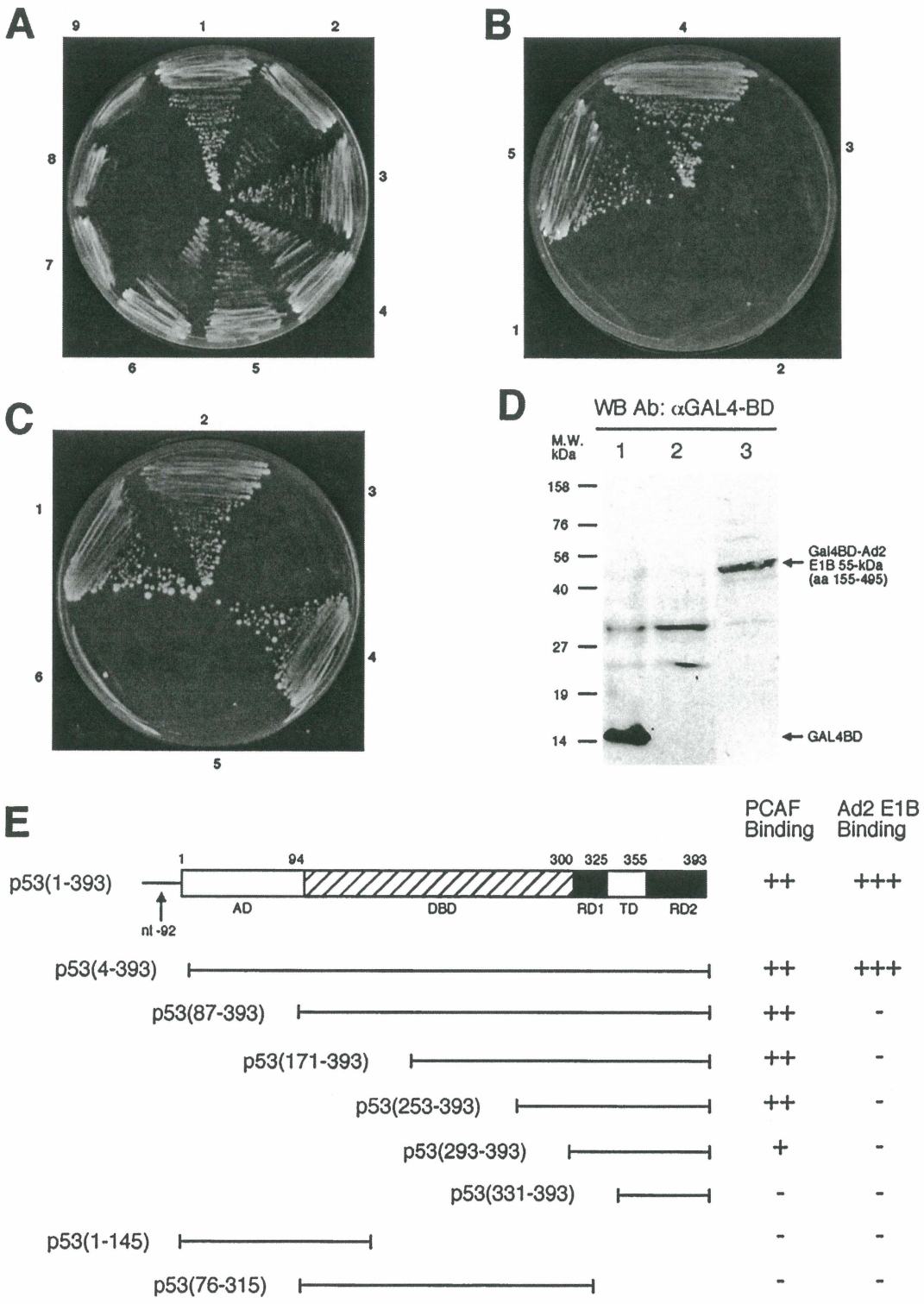

FIG. 7. Yeast two-hybrid assays of p53, PCAF, and E1B interactions. (A) PCAF binds to the C-terminal domain of p53. Yeast cells were transformed with a hybrid between full-length PCAF and GAL4 BD and various GAL AD hybrids containing p53 (1–393) (sector 1), p53 (4–393) (sector 2), p53 (87–393) (sector 3), p53 (171–393) (sector 4), p53 (253–393) (sector 5), p53 (293–393) (sector 6), p53 (331–393) (sector 7), p53 (1–145) (sector 8), and p53 (76–315) (sector 9), and the transformed cells were grown under His^- selection in the presence of 5 mM 3-AT. (B) The central region of Ad2 E1B 55-kDa protein interacts with p53. Yeast cells were transformed with p53 (1–393)-GAL4 AD hybrid and various hybrids between GAL4 BD and different domains of Ad2 E1B 55-kDa protein: aa 437 to 495 (sector 1), aa 1 to 161 (sector 2), aa 155 to 437 (sector 3), aa 155 to 495 (sector 4), and aa 1 to 437 (sector 5). The transformed cells were grown under His^- selection as for panel A. (C) The N-terminal domain of p53 is necessary for binding to Ad2 E1B 55-kDa protein. A fusion between GAL4 BD and Ad2 E1B (aa 155 to 495) (sectors 1 to 3) or full-length Ad12 E1B (sectors 4 to 6) was introduced into yeast along with GAL4 AD hybrids containing p53 (1–393) (sectors 1 and 6), p53 (4–393) (sectors 2 and 5), p53 (87–393) (sector 3), and Ad12 E1B full-length protein (sector 4). (D) Expression of GAL4 BD–E1B 55-kDa protein hybrids. The E1B-GAL4 BD hybrids were detected using anti-GAL4 BD antibody (Santa Cruz Biotechnology). Lane 1 shows lysates of yeast harboring only GAL4 BD plasmid and serves as control. Lanes 2 and 3 were yeast cell extracts prepared from yeast cells that contained GAL4 AD-p53 (1–393) hybrid and one of the GAL4 BD–E1B hybrids: full-length Ad12 E1B

fused with GAL4 BD and AD, and they were used for two-hybrid assays. As shown in Fig. 7B, C, and E, two overlapping constructs spanning residues 1 to 437 and 155 to 495 exhibited strong interactions with p53 (Fig. 7B, sectors 4 and 5). However, Ad2 E1B 55-kDa protein carrying residues 1 to 161 (Fig. 7B, sector 2) and 437 to 495 (sector 1) did not bind to p53. Thus, the central portion of Ad2 E1B 55-kDa protein binds to the N terminus of p53 (inability of the Ad2 construct containing aa 155 to 437 to bind to p53 was due to the failure of this construct to express itself in yeast [see Fig. 8C, lane 4]). Both p53(1–393) and p53(4–393) constructs exhibited interaction with the Ad2 E1B (aa 155 to 495) construct (Fig. 7C, sectors 1 and 2). However, p53 constructs lacking the N-terminal domain, such as p53(87–393) (Fig. 7C, sector 3), and other p53 constructs shown in Fig. 7E (data not shown) did not show interaction with Ad2 E1B. Curiously, p53(1–145) fused to GAL4 AD did not bind to Ad2 E1B, although in biochemical assays, aa 1 to 123 of murine p53 were shown to be sufficient for binding to Ad2 E1B (24). This could stem from altered conformation of this p53-GAL4 AD hybrid. Collectively, these results indicate that the amino-terminal domain of p53 is required for binding to the central portion of Ad2 E1B, in full agreement with previous reports (33, 70). However, we were unable to detect an interaction between p53 and Ad12 E1B 55-kDa protein (Fig. 7C, sectors 5 and 6), although Ad12 E1B 55-kDa protein self-interaction was detected (sector 4). This may be explained by the extremely low expression level of Ad12 E1B-GAL4 BD fusion, being virtually undetectable in Western blots (Fig. 7D, lane 2), while Ad2 E1B-GAL4 BD fusion can be detected (lane 3).

A domain near the C terminus of Ad2 E1B 55-kDa protein is necessary for binding to PCAF. We also tested PCAF-E1B interaction using the two-hybrid assay. Five Ad2 E1B 55-kDa protein constructs containing various regions of the protein were used (Fig. 8A). As shown in Fig. 8B, only the Ad2 55-kDa protein construct carrying residues 155 to 495 exhibited interaction with PCAF (Fig. 8B, sector 4), although all these Ad2 E1B constructs except construct 3 containing aa 155 to 437 were well expressed in yeast (Fig. 8C). As both Ad2 E1B constructs 4 and 5 bind to p53 (Fig. 7), these results appear to suggest that the PCAF-binding site of E1B is distinct from the p53-binding site shown on the top of Fig. 8A. Thus, a unique region near the C terminus of Ad2 E1B 55-kDa protein is likely to be necessary for binding to PCAF.

E1B 55-kDa protein interferes with PCAF-p53 interaction. One possible mechanism by which E1B 55-kDa protein inhibits acetylation of p53 by PCAF might be that PCAF fails to interact with p53 in the presence of E1B. To investigate this possibility, we employed a reverse two-hybrid assay. PCAF-GAL4 BD hybrid and p53-GAL4 AD fusion were introduced into yeast together with pCu424 expressing residues 1 to 437 of the Ad2 E1B 55-kDa protein (again, the WT Ad2 E1B could not be cloned in this plasmid) or the full-length Ad12 E1B 55-kDa protein. Since p53 and PCAF can interact with each other, yeast growth would be expected in medium lacking histidine in the presence of p53-GAL4 AD and PCAF-GAL4 BD hybrids. If E1B interferes with p53-PCAF interaction, the His^+ phenotype might be lost when E1B-expressing pCu424 is present in addition to p53-GAL4 AD and PCAF-GAL4 BD hybrids. Indeed, as shown in Fig. 9A, Ad2 E1B 55-kDa protein (aa 1 to 437) expression led to the loss of the His^+ phenotype in the presence of PCAF-GAL4 BD hybrid along with p53(1–393)-GAL4 AD fusion (Fig. 9A, sector 4). In contrast, when the DNA fragment encoding Ad2 E1B (1–437) was cloned in reverse orientation in pCu424, significant yeast growth was seen (Fig. 9A, sector 1). Failure of yeast growth in the presence of Ad2 E1B cannot be ascribed to simple toxicity of E1B to yeast, as yeast containing Ad2 E1B constructs grows very well in nonselective media, and the E1B expression in yeast can be detected (Fig. 9B). Interestingly, the His^+ phenotype was retained when pCu424-Ad12 E1B was introduced into yeast along with p53-GAL4 AD and PCAF-GAL4 BD (Fig. 9A, sector 3). As a negative control, the p53 hybrid was cotransformed with empty vectors pGBDU and pCu424. As expected, no yeast growth was detected (Fig. 9A, sector 2). These results indicate that Ad2 E1B 55-kDa protein interferes with p53-PCAF interaction. Ad2 E1B was expressed when cloned in correct orientation (Fig. 9B, lane 3). The GAL4 BD-PCAF fusion was also expressed in all cases (Fig. 9C). However, we were unable to detect Ad12 E1B expression from pCu424-Ad12 E1B plasmid. Thus, the failure of pCu424-Ad12 E1B to prevent p53-PCAF interaction (Fig. 9A, sector 3) might reflect a low level of expression of Ad12 E1B 55-kDa protein in yeast (also Fig. 7D, lane 2).

DISCUSSION

Key to its function as a tumor suppressor, p53 regulates the expression of a specific set of genes involved in cell growth control. p53 activates transcription of genes containing specific DNA-binding sites for p53. Under normal physiological conditions, p53 exists in low abundance, apparently in an inactive, latent state with low sequence-specific DNA-binding activity (29). Multiple types of modifications occur on p53 in response to DNA damage or other genotoxic stresses. Covalent modifications of p53, such as phosphorylation by kinases, dephosphorylation by phosphatases, and acetylation by acetylases, lead to its stabilization (53), specific interaction with other regulatory proteins such as 14-3-3 (64), and enhanced DNA-binding activity (17, 35, 47). Consequently, any interference with covalent modifications of p53 could compromise its functions. The results presented here show that the E1B 55-kDa protein specifically inhibits p53 acetylation by PCAF both in vitro and in vivo (Fig. 2 and 3). Since acetylation of p53 has been shown to enhance its sequence-specific DNA-binding activity and also to be induced in response to DNA damage (17, 35, 47), the E1B oncoprotein represses p53 functions at least in part by inhibiting its acetylation. Indeed, we have found that the DNA-binding activity of p53 in cells expressing E1B 55-kDa protein is greatly reduced (Fig. 4).

How does E1B specifically inhibit acetylation of p53 by PCAF? We found that E1B interacts with both p53 and PCAF (Fig. 5 to 8). In addition, p53 also binds to PCAF (Fig. 7). Interestingly, E1B can efficiently prevent the interaction between p53 and PCAF (Fig. 9), suggesting that E1B may inhibit the acetylation of p53 by PCAF by blocking the enzyme-substrate interaction. Consistent with this, E1B does not affect the acetylation of histones by PCAF (Fig. 2). Our data indicate

(lane 2) and Ad2 E1B (aa 155 to 495) (lane 3). (E) Summary of p53-PCAF and p53-E1B interaction. Relative activities of reporter gene expression as measured by growth under His^- selection in the presence of 5 mM 3-AT (A to C) or β-galactosidase expression (data not shown) are illustrated at the right. β-Galactosidase activities were assessed with in situ X-Gal staining on plates or quantitative measurement using Galacto-Light reagents (Tropix) and a luminometer (data not shown). The p53 domains are denoted as in Fig. 1. WB, Western blot; Ab, antibody; nt, nucleotide.

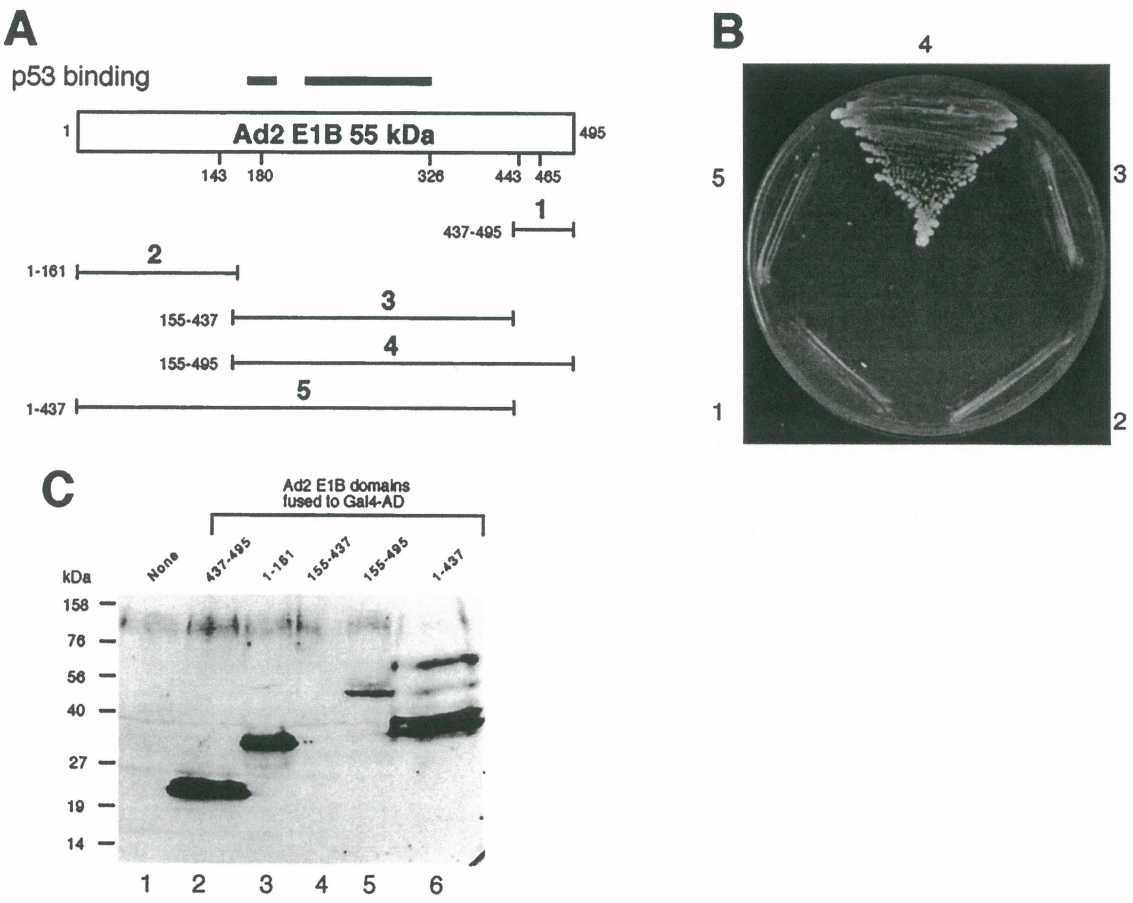

FIG. 8. Mapping of the domain of Ad2 E1B 55-kDa protein required for binding to PCAF. (A) Schematic drawings of different Ad2 E1B fragments fused to GAL4 AD. The regions required for interacting with p53 are denoted with heavy lines on the top of the full-length Ad2 E1B 55-kDa protein. The Ad2 E1B fragments are numbered from 1 to 5, and the residues contained in each construct are indicated on the left. (B) The domain near the C terminus of Ad2 E1B is required for PCAF binding. The PCAF-GAL4 BD hybrid was tested for its interaction with Ad2 E1B. The GAL4 AD was fused to different Ad2 E1B fragments as shown in panel A: aa 437 to 495 (sector 1), aa 1 to 161 (sector 2), aa 155 to 437 (sector 3), aa 155 to 495 (sector 4), and aa 1 to 437 (sector 5). (C) Expression of GAL4 AD-Ad2 E1B hybrids. The hybrids in yeast cell extracts were detected using anti-GAL4 AD antibody (Santa Cruz Biotechnology). The Ad2 E1B domain in each hybrid is indicated on the top of each lane. Lane 1 shows yeast lysate without any GAL4 AD hybrid.

that Ad2 E1B fragment aa 1 to 437 is able to prevent p53-PCAF interaction (Fig. 9). Since this E1B fragment binds to p53 but not to PCAF (Fig. 7 and 8), binding of E1B to p53 may be sufficient for E1B to interfere with the p53-PCAF interaction. Nonetheless, E1B 55-kDa protein also binds to PCAF in vitro and in vivo (Fig. 5 and 8). Thus, by association with both p53 and PCAF, E1B protein might shift equilibrium of protein-protein complexes from p53-PCAF to p53-E1B and PCAF-E1B, thereby effectively sequestering p53 and PCAF and preventing PCAF from acetylating p53. It appears that PCAF and p53 bind to different sites in E1B (Fig. 7 and 8). This finding might permit an assessment of the relative importance of p53-E1B and PCAF-E1B interactions in inhibiting acetylation of p53 through extensive mutagenesis of the p53- and PCAF-binding sites in the E1B 55-kDa protein.

While the role of E1B-PCAF interaction in inhibiting acetylation of p53 is not clear at present, blocking the catalytic core of PCAF (the HAT domain) by E1B is unlikely to be the cause of inhibition; rather, binding of E1B to PCAF might induce conformational change of PCAF so that its substrate specificity is altered. We have found that a mutant PCAF lacking aa 62 to 464 can acetylate both p53 and histones because it still contains the HAT domain (44). Interestingly, acetylation of p53 but not that of histones by this mutant PCAF is also inhibited by E1B (data not shown). Thus, binding of E1B to a domain in the C-terminal portion of PCAF may play a role in the observed inhibition of p53 acetylation, as this mutant PCAF lacks most of the sequence in its N-terminal region. Future study will address which domain of PCAF is responsible for binding to E1B 55-kDa protein.

The acetylation of p53 by PCAF is severely impaired in cells expressing E1B 55-kDa protein (Fig. 3). Accumulation of acetylated p53 occurs normally in G401 cells upon treatment with the specific deacetylase inhibitor TSA (Fig. 3B). By contrast, acetylated p53 is virtually undetectable under the same conditions in cell line G401-CC3, derived from G401 cells by stable transfection with an Ad12 E1B 55-kDa protein-expressing vector (55). The acetylation of p53 in 293 cells is also suppressed. While 293 cells express both E1A and E1B proteins, G401-CC3 cells express only E1B 55-kDa protein. Therefore, E1B 55-kDa protein is most likely to be responsible for the observed inhibition of p53 acetylation by PCAF, consistent with the in vitro studies (Fig. 2). Effects of E1A oncoproteins on the activities of PCAF, p300, and CBP have been reported previously (1, 5, 19, 44). While E1A was found to bind to PCAF (44), it does not appear to affect the HAT activity of PCAF. Instead, E1A protein was found to stimulate HAT activities of p300 and CBP under certain circumstances (1).

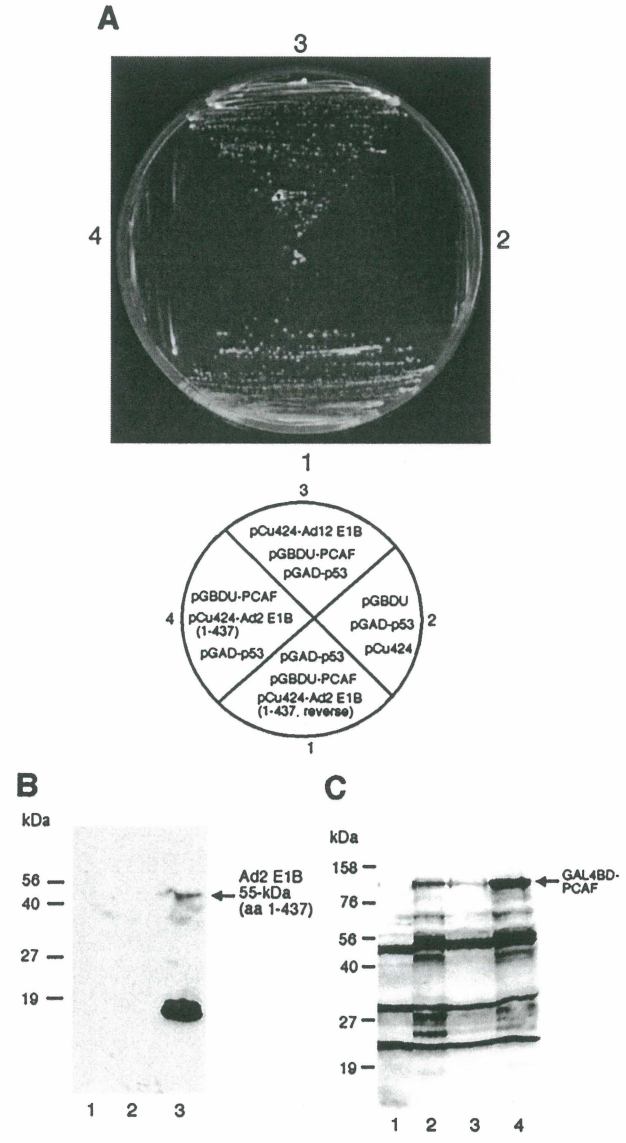

FIG. 9. E1B 55-kDa protein interferes with the PCAF-p53 interaction. (A) For reverse two-hybrid assay, full-length PCAF-GAL4 BD hybrid and p53(1–393)-GAL4 AD hybrid were introduced into yeast cells together with pCu424 containing the gene for Ad2 E1B 55-kDa protein (aa 1 to 437) cloned in reverse orientation (sector 1) or in correct orientation (sector 4) or the gene for WT Ad12 E1B 55-kDa protein in correct orientation (sector 3). Sector 2 serves as a negative control, in which the same yeast strain was transformed with pGBDU-C3, pGAD-p53, and pCu424. The plasmids used in each transformation shown on the top are depicted below. (B) Expression of Ad2 E1B (aa 1 to 437). Extracts were prepared from yeast cells that were transformed with pGAD-p53, pGBDU, and pCu424 (lane 1); pGAD-p53, pGBDU-PCAF, and pCu424-Ad2 E1B (1 to 437, reverse) (lane 2); and pGAD-p53, pGBDU-PCAF, and pCu424-Ad2 E1B (1 to 437) (lane 3). The extracts were then subjected to Western blot analysis with antibody 2A6 against Ad2 E1B 55-kDa protein. Note that Ad2 E1B was expressed only when the gene was cloned in the correct orientation (lane 3). The dark band in lane 3 may be partially degraded Ad2 E1B, as this band was absent in lanes 1 and 2. (C) Expression of PCAF-GAL4 BD hybrid. The hybrid was detected with anti-GAL4 BD antibody. The plasmids used for transformation were pGAD-p53, pGBDU, and pCu424 (lane 1); pGAD-p53, pGBDU-PCAF, and pCu424-Ad2 E1B (1 to 437) (lane 2); pGAD-p53, pGBDU-PCAF, and pCu424-Ad12 E1B (lane 3); and pGAD-p53, pGBDU-PCAF, and pCu424-Ad2 E1B (1 to 437, reverse) (lane 4).

Conversely, recent studies demonstrated that the E1A oncoproteins may repress HAT activity of both PCAF and p300 in vitro (5, 19). Regardless of the potential role of E1A proteins in regulation acetylation, our results clearly indicate that E1B 55-kDa protein inhibits acetylation of p53 by PCAF both in vivo and in vitro.

Whereas acetylation of p53 enhances its sequence-specific DNA-binding activity (17, 35, 47), a number of other modifications of the p53 C-terminal domain can also activate its DNA-binding function (20). We found that anti-p53 antibody PAb421 can effectively enhance p53 DNA binding in G401-CC3 nuclear extracts (Fig. 4), despite reduced p53 acetylation in this cell line (Fig. 3). Thus, it is conceivable that binding of PAb421 to the C-terminal domain of p53 may have a similar effect on activating p53 DNA-binding activity as acetylation of the lysine residues within the p53 C-terminal domain. Interestingly, we found that PAb421 cannot activate p53 DNA binding in 293 nuclear extracts (Fig. 4), suggesting that E1A may have additional inhibitory effects on p53 DNA-binding activity. Indeed, p53 appears to form high-molecular-weight oligomers when E1A proteins are expressed in G401 cells (54). Such modified p53 protein may be less competent for DNA binding, as its transcriptional transactivation function was greatly repressed by E1A (54).

The E1B 55-kDa protein represses transcriptional transactivation by p53 by binding directly to DNA-bound p53 without destabilizing p53-DNA complexes, thereby tethering the E1B transcriptional repression domain to promoters containing p53 binding sites (71). Furthermore, E1B 55-kDa protein appears to enhance p53-DNA interaction and could repress transactivation mediated by p53 in an in vitro assay using purified RNA polymerase II components (39, 40). We show here that E1B 55-kDa protein inhibits effectively p53 acetylation. Inhibition of p53 acetylation (thus reducing its affinity to its binding sites [Fig. 4]) and direct targeting of DNA-bound p53 might reflect two levels of repression of p53 functions by E1B. At the first level, E1B represses acetylation of p53 by PCAF, and possibly also by p300 and CBP. At the second level, E1B could still target any p53 that binds to specific sites within target promoters due to incomplete inhibition of acetylation or other means, thereby resulting in direct transcriptional repression. The two-level, fail-safe repression mechanisms on p53 suggest that E1B 55-kDa oncoprotein is a particularly powerful repressor of p53. Whether E1B also inhibits other types of covalent modifications occurring on p53 remains to be established. Our unpublished data indicated that phosphorylation of p53 at serine 392 was not affected by E1B 55-kDa protein in a Western blot analysis using a specific antibody against p53 phosphorylated at serine 392.

The Ad E1A and E1B proteins play important roles in cell transformation (3, 45). The E1A oncoproteins stimulate cell proliferation by binding to pRB, p300, and CBP (reviewed in reference 11). The E1B 55-kDa protein acts in cell transformation by inactivating the p53 pathway. Although inactivation of pRB and p53 pathways is essential to the transformation of a normal cell into a tumor cell (18), deregulation of other cellular regulatory circuitry may also be involved in cell transformation and development of cancer. As PCAF is implicated in regulation of cell differentiation, cell cycle progression, and transcriptional regulation (43, 50, 62), deregulation of the PCAF pathway might also play a role in cell transformation. Intriguingly, just as E1A and E1B 55-kDa proteins inhibit p53 transactivation function, the same viral oncoproteins bind to PCAF, although it is likely that E1A and E1B affect different aspects of the PCAF functions. Furthermore, E1A appears to compete with PCAF for access to p300 (68), which could affect critical functions of PCAF in regulating cellular pathways. Additionally, the Tax oncoprotein of the human T-cell leukemia virus type 1 recruits PCAF for transactivating viral promoters (23). Such exploitation of PCAF by viral oncoproteins might

perturb cellular physiology, thus contributing to cell transformation.

Increasing evidence supports critical roles for protein acetylation in cellular physiology, including regulation of protein-DNA and protein-protein interaction, as well as protein stability. Like phosphorylation, acetylation can regulate key cellular processes in response to extracellular signals. Thus, it has been proposed previously that acetylation as a biologically relevant modification may be as important as phosphorylation (26). Consistent with this view, our data demonstrate that the Ad E1B 55-kDa oncoproteins specifically inhibit p53 acetylation by PCAF, while its histone acetylation and autoacetylation activities were not affected. These data suggest that the inhibition of p53 acetylation by viral proteins may represent an important mechanism of p53 inactivation. Future investigation on how E1B 55-kDa oncoprotein affects PCAF functions will provide insight into the biological functions mediated by PCAF, as well as how modulation of cellular acetylase activities by viral oncoproteins contributes to cell transformation and oncogenesis.

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Chapter 3 Article 2

Yue Liu, Lisa Y. Zhao, XiangJiao Yang and Daiqing Liao.
PCAF acetylates p73 and serves as a coactivator of p73-mediated transactivation (submitted)

As the first author of this article, I am a main contributor to most of the data presented in this article. I made all the p73 mutants containing Lys to Arg mutation(s), purified most of the proteins, and performed the yeast two hybrid assay, immunoprecipitation assay and GST pull down assay (Fig. 1, 2). I also did all the in vitro and in vivo acetylation assays (Fig. 3), the transient reporter assay (Fig. 4, 6) as well as the apoptotic assay (Fig. 5) and the colony formation assay (Fig. 5, 6).

PCAF acetylates p73 and serves as a coactivator of p73-mediated transactivation

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Running title: Acetylation and coactivation of p73 by PCAF

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Abstract

The tumor suppressor p53 homologue p73 shares significant amino acid sequence identity with p53. Like p53, p73 recognizes canonical p53 DNA-binding sites and activates p53-responsive target genes and induces apoptosis. Moreover, transcription coactivator and acetylase p300/CBP binds to and coactivates with both p53 and p73 in stimulating the expression of p53-target genes. Here we show that the acetylase and coactivator PCAF binds to a central domain of p73 that is shared by all p73 isoforms. PCAF also interacts with a small C-terminal region that is unique to p73α. This small C-terminal region is acetylated by PCAF in vitro. We determined that lysine 623 of p73α is a PCAF acetylation site. p73α but not p73β is also acetylated in vivo. We further show that PCAF significantly stimulates both p73α and p73β-dependent transcription and apoptosis. Although PCAF does not acetylate p73β, PCAF histone acetyltransferase (HAT) domain is required for PCAF to stimulate p73β-mediated transactivation. Interestingly, coactivation by PCAF is diminished using p73α mutant lacking the acetylation site. Collectively, these data suggest that p73 recruits PCAF to stimulate the transcription of p73 target genes and PCAF's acetylation of p73α may regulate its activity.

Introduction

p53 is a tumor suppressor whose inactivation is a precondition to most human cancers (29, 42, 56). p53 plays a central role in regulating cell growth, particularly in response to various forms of stress including DNA damage and viral infection. p53 sits on a critical node of signal transduction networks that control cell growth and death. p53 exerts its tumor suppressor function, to a large extent, by regulating expression of genes that control cell cycle progression

and apoptosis (57, 61, 66). Three specific domains of p53 contribute to its transactivation activity: the N-terminal transactivation domain (TAD), the central sequence-specific DNA-binding domain (DBD) and the C-terminal regulatory domain (RD). The TAD contacts several transcriptional regulators, including components of the RNA polymerase II transcriptional machinery, such as TAFIID, TAFIIH and the transcriptional coactivators p300 and CBP. The DBD recognizes and binds to a specific consensus sequence, four copies of pentamer PuPuPuC(A/T) oriented in alternating directions (7), which has been identified in an increasing number of p53 target genes (50, 61, 66). The RD regulates p53 transcriptional activity. Posttranslational modification of this region, including stress signal-induced acetylation, enhances p53 stability, DNA-binding and transactivation activities (13, 20, 31, 46). Cellular acetylases p300, CBP and PCAF can acetylate specific lysine residues of p53 within RD, and MDM2, a negative regulator of p53, and adenovirus E1B 55-kDa oncoprotein were shown to inhibit acetylation of p53 by p300/CBP and PCAF (20, 26, 32). Thus acetylation of p53 may play an important role in regulating p53's biochemical function.

Several p53-like proteins including the p73 and p63 families of proteins have been reported in recent years (59). These proteins resemble p53 in both sequence and function. Like p53, p73 contains TAD in its N-terminal domain, DBD in the central portion, followed by an oligomerization domain (OD), and ends with a potential regulatory domain in the C-terminal region (2, 24, 34, 59)(also see Fig. 2). p73 can binds to the consensus p53-binding DNA and activates expression of genes containing such sequence as well as induction of apoptosis (1, 10, 19, 24, 33, 62). p300/CBP is a coactivator of both p53 and p73 (3, 13, 64). One conspicuous difference between p53 and p73 is that p53 gene gives rise to only one product, whereas p73 gene produces over six variants (24, 34, 59). All these p73 isoforms retain the DBD and OD, but

alternative splicing of p73 mRNA results in isoforms that have different C-terminal truncations (23, 59). Unlike p53, which is phosphorylated by DNAPK and ATM (25, 42), p73 protein accumulates and undergoes c-Abl-dependent tyrosine phosphorylation in response to DNA damage (1, 10, 62). Furthermore, p73 is not targeted for inactivation by viral oncoproteins such as simian virus 40 T antigen, adenovirus E1B 55-kDa and human papillomavirus E6 (12, 16, 33, 41, 44, 58), although MDM2 can inhibit p73-mediated transactivation (26).

The extended C-terminal region of p73α, which is not present in p53, has a motif similar to SAM (sterile alpha motif). This motif is involved in protein-protein interactions (2). It has been shown that the C-terminal region of p73α may negatively regulate p53-like activities of p73. For example, p73β, which lacks much of the extended C-terminal domain including SAM, is more active as transcriptional transactivator of p53-responsive reporter genes (6, 36). As a protein-protein interaction module, SAM can potentially recruit proteins that modulate p73α functions. In addition, there are ~70 amino acids beyond SAM (post-SAM domain) and they may also be involved in regulating p73α activities. Indeed, deletion of post-SAM domain from p73α enhances its transactivation potential (39).

p53 C-terminal domain also negatively modulates its DNA-binding and transactivation activity (18), and covalent modifications including acetylation and SUMO-1 conjugation within this region can relieve such effect (13, 31, 45, 46). p73α is also modified by SUMO-1 at lysine 627, although such modification does not appear to affect its transactivation potential (36). Up to date, whether p73 is subjected to acetylation is unknown.

p73 as a DNA-binding transcriptional activator is clearly demonstrated. However, the mechanism by which p73 mediates transactivation remains unclear. For example, although p300/CBP was shown to act as a coactivator of p73-mediated transactivation (64), the HAT

function of p300/CBP appears to be dispensable (63). Thus, it remains possible that p73 recruits a different coactivator for targeted histone acetylation required for transcription activation, while p300/CBP could play a different role in stimulating p73-dependent transcription such as by bridging p73 and basal transcription machinery. In addition, there are multiple forms of p73 and their activities could be differentially regulated. We have begun to address some of these issues. In this study, we showed that p73α is acetylated by PCAF. We determined that lysine 623 at the p73α C-terminus is required for acetylation within this region. PCAF binds to a region of p73 that is present in all p73 isoforms and strongly stimulates its transactivation activity. In addition, we showed that the HAT domain of PCAF and lysine 623 of p73α, the acetylation site, are important for coactivation of p53-responsive reporter genes and the induction of apoptosis. The results indicate that PCAF is a p73 coactivator, for which the HAT activity of PCAF is required. We also suggest that acetylation of p73α may be important in regulating its biochemical function.

MATERIALS AND METHODS

Plasmids. The pcDNA3-HA-p73α and pcDNA3-HA-p73β plasmids were obtained from M. Kaghad. PG13-Luc and pWAF1-Luc were provided by B. Vogelstein. pGBDU-PCAF, pGAD-p73α, pGAD-p73β, pGEX-4T-1-p73α, pGEX-4T-1-p73αN (aa 1-249, F1), pGEX-4T-1-p73αM (aa 260-430, F2), pGEX-4T-3-p73αS(aa 430-560, F3) and pGEX-4T-3-p73αC(aa 560-636, F4), pCX-Flag-PCAF, pCX-Flag-PCAFΔHAT1(aa 579-608 were deleted) and pCX-Flag-PCAFΔHAT2 (aa 608-623 were deleted) were constructed. Site-specific mutations that convert K to R were produced by PCR-directed mutagenesis using pcDNA-HA-p73α as template, and the mutants p73αC-K620-623-627R (K3R), K623R, K620R, K623-627R, and K627R were

cloned into pGEX-4T-3 for producing GST-fusion proteins, and HA-p73α-K3R, -K623R and -K620R were cloned into pcDNA3.1. All but the C-terminal 13 residues after K627 were included in HA-p73α mutants. All mutants were confirmed by DNA sequencing.

Cell culture and Antibodies. Human cervical carcinoma HeLa and human osteosarcoma SaoS-2 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Small-cell lung carcinoma H1299 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The anti-HA and -Flag mouse monoclonal antibodies were purchased from Roche Molecular Biochemicals and Sigma, respectively. The anti-p73 rabbit polyclonal antibody (H-79) and anti-GFP antibody were purchased from Santa Cruz Biotechnology. The anti-acetylated lysine antiserum was purchased from Upstate Biotechnology.

Purification of GST-p73 fusion proteins. The glutathione S-transferase (GST)-p73α fusion proteins were expressed and purified from *E. coli*. Briefly, bacterial pellet in cold phosphate-buffered saline (PBS, pH 7.4) was sonicated, and Triton X-100 was added to the suspension to 1%. The mixture was incubated for 15 min and then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through 0.2 μm filter and then mixed with glutathione Sepharose 4B resin (Amersham-Pharmacia) that was washed with PBS, and the mixture was rotated for at least 2 h at 4°C. The resin was collected and washed for four times with PBS and the fusion protein was eluted with elution buffer (7.5 mM reduced glutathione in PBS).

GST pull down assay. The purified Flag-tagged PCAF was incubated with glutathione-Sepharose 4B beads (50% slurry) containing approximately 0.5 μg of various GST-p73 fusion proteins in PBS. One hour after incubation at 4°C, the mixtures were washed three times with PBS and once with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate [SDS], and 1% [wt/vol] sodium deoxycholate). Bound proteins were analyzed on a 10% SDS-polyacrylamide gel and detected by Western blot (WB) using the anti-Flag antibody to assay p73-PCAF interaction and to map the PCAF binding site on p73.

Coimmunoprecipitation (IP) and Western blot (WB) analysis. In each IP experiment, approximately 0.5 to 1 μg of each purified protein was incubated with an appropriate antibody in buffer B (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 150 mM KCl) with protease inhibitors (10 mg/ml pepstatin, 5 mg/ml leupepstatin, 20 mg/ml aprotinin, and 1 mM PMSF) for at least 1 h with rotation at 4°C. The amount of antibody used per IP assay is different depending on specific antibodies; the typical amount is 1 μg. Protein G agarose beads (30 μl; Roche Molecular Biochemicals) were added into the protein-antibody mixture and incubated at 4°C for 1 h with rotation. The beads were collected by centrifugation and washed 3 times with buffer B. The beads were collected by centrifugation and mixed with 30 μl of 1X SDS loading buffer. The precipitated proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in 25 mM Tris base-190 mM glycine at 50 V for 2 h at 4°C. The coprecipitated proteins were detected using an appropriate antibody with the enhanced chemiluminescence (ECL, Amersham-Pharmacia) kit.

To detect p73-PCAF interaction in vivo, SaoS-2 or HeLa cells were transfected with 2 μg PCX-Flag-PCAF along with 2 μg of pcDNA3, pcDNA3-HA-p73α or pcDNA3-HA-p73β using

Superfect reagent (Qiagen). Briefly, Superfect-DNA mixture was layered onto the cells for 3 h, and then replaced with fresh complete DMEM. Cells were harvested 48 h posttransfection. Cells were lysed with buffer B, and the lysates were precleared with 20 μl of protein-G agarose and then incubated for 3 h at 4°C with fresh protein-G beads and 2 μg of anti-HA (for HA-tagged p73) or anti-Flag (for Flag-tagged PCAF). The beads were collected by centrifugation and washed 4 times with RIPA buffer. The precipitates were analyzed with SDS-PAGE and WB.

Yeast two hybrid assay. p73α and p73β gene were fused to Gal4 activation domain (AD) in plasmids pGAD-C(x), and PCAF was fused with Gal4 DNA-binding domain (BD) in pGBDU-C(x). The yeast strain PJ69-4A was used for two hybrid assay as described previously (21). The pGAD-p73 and pGBDU-PCAF were introduced into the host yeast by the standard lithium acetate transformation method. To test potential protein-protein interaction, transformants were screened for growth in medium lacking histidine but in the presence of 5 mM 3-aminotriazol (3-AT) (His+ phenotype) or lacking adenine (Ade+ phenotype).

Acetyltransferase assay. The human PCAF was purified as described previously and used as acetylase (60). Purified p73 and its different fragments were subjected to acetylation by PCAF. Protein samples were incubated at 30°C for 30 min in a total volume of 20 μl in acetylation buffer (50 mM Tris-HCl, pH 8.0, 10% Glycerol, 1 mM 1,4-dithiothreitol, 1 mM PMSF, 0.1 mM EDTA, 10 mM sodium butyrate) with 90 pmol of 1-^{14}C-acetylcoenzyme A (55 mCi/mmol; Amersham Pharmacia). The reaction mixtures were then analyzed by SDS-PAGE, and the gels were stained with Coomassie brilliant blue, dried, and were subjected to autoradiography (3 to 6 days).

To detect the in vivo p73 acetylation by PCAF, SaoS-2 cells were transfected with 2 μg pCX-Flag-PCAF along with 2 μg of pcDNA3, pcDNA3-HA-p73α or pcDNA3-HA-p73β as described above. Cells were washed with PBS 36 h posttransfection, and then grown in complete DMEM supplemented with the deacetylase inhibitor trichostatin A (TSA; Sigma) at a final concentration of 5 μM. Two hours after TSA treatment, cells were washed twice with ice-cold PBS containing 5 μM TSA and lysed on ice in buffer B containing 5 μM TSA. Lysates were subjected to IP with anti-HA against HA-tagged p73, and acetylated HA-p73 was detected by rabbit anti-acetylated lysine antiserum in WB.

Luciferase assay. SaoS-2 cells (50% confluent) were transfected with the PG13-firefly luciferase (Luc) reporter which contains multiple copies of the p53-binding sequence, or pWAF1-Luc, which contains a fragment from the p21/CIP1/WAF1 gene promoter (8), together with a CMV promoter-driven Renilla luciferase (RLuc) reporter plasmid (1 μg) and a combination of different plasmids as indicated in various figures using Superfect reagent (100 ng of p73 plasmids and 1 μg of PCAF plasmids). Irrelevant plasmids were added so that the total amount of DNA transfected was kept the same in each transfection. Cells were harvested for luciferase assays 18 h posttransfection using dual luciferase assay regents (Promega) according to the protocol supplied by the manufacturer. Firefly luciferase activity was normalized against RLuc activity.

Apoptotic assay. SaoS-2 cells (40% confluent) were transfected with the pEGFP plasmid (1 μg) together with a combination of various expression plasmids as indicated in different experiments (500 ng of p73 plasmids, 1 μg of PCAF plasmids). Empty vectors were used as controls. The

morphology of cells expressing GFP was analyzed under a fluorescence microscope 32 h after transfection. Apoptotic cells were identified by their rounded and shrunken morphology in contrast to the spread-out appearance of non-apoptotic cells, counted on a blind basis, and presented as a percentage of the total population of fluorescent cells.

Colony formation assay. H1299 cells (40% confluent) were transfected using Superfect reagent with a combination of various plasmids as indicated in specific assays (500 ng of p73 plasmids, 1 μg of PCAF plasmids). Equal dose of neomycin resistant gene in each transfection was strictly maintained. Cells were split in triplicate and grown under G418 (0.4 mg/ml) selection. G418-resistant colonies were selected by growth in complete RPMI 1640 medium containing G418 for two weeks. The G418-resistant colonies were then stained with Giemsa solution (Sigma) and counted. The experiments were repeated at least three times in duplicate.

RESULTS

p73 binds to PCAF in vitro and in vivo. Yeast two hybrid assays were employed to test the interaction between p73α, β and PCAF. Presence of either Gal4-AD-p73α or Gal4-AD-p73β with Gal4-BD-PCAF in yeast led to its growth on medium lacking histidine, but not when Gal4-AD-p73 and empty Gal4-BD plasmids were transformed into yeast. Conversely, Gal4-BD-PCAF did not interact with Gal4-AD (Fig. 1A). Thus, p73α and p73β bind to PCAF specifically in yeast.

To confirm these results, we examined the p73-PCAF interaction using IP and GST pull-down assays with purified recombinant GST-p73 and PCAF proteins. We performed IP by incubating anti-GST antibody, protein G agarose and equal amounts of GST or GST-p73α.

Precipitated proteins were analyzed by Western blot. As seen in Fig. 1B, PCAF is coprecipitated with GST-p73α fusion (lane 1), but not with GST (lane 2), indicating a direct interaction between p73 and PCAF in vitro. We then examined PCAF-p73 interaction in vivo. Plasmids HA-p73 and Flag-PCAF were transfected into Soas-2 and HeLa cells. Fig. 2C indicates that HA-p73α was precipitated with Flag-PCAF using anti-Flag antibody (lane 4), but not without Flag-PCAF cotransfection (lane 3). Lane 1 shows the expression of HA-p73α in transfected cells. Conversely, PCAF was coprecipited with both HA-p73α and HA-p73β (Fig. 1D, lanes 4 and 5), and anti-HA antibody did not recognize PCAF (lane 3). Lane 1 showed the purified Flag-PCAF. The presence of HA-p73α and –β in the immunoprecipitates is shown in the lower portion of Fig. 1D. Thus, PCAF binds to p73α and –β in vitro and in vivo.

PCAF binds to the central region of p73 and a unique p73α C-terminal domain. Having determined that PCAF binds to PCAF, we then mapped its binding site in the p73 protein. Four GST-p73 fusion constructs that cover the entire p73α sequence were made (Fig. 2A), and the recombinant proteins were expressed and purified from *E. coli*. These GST fusion proteins were used for GST pull-down assays. Bound Flag-PCAF was analyzed in Western blot analysis with antibody to the Flag tag. PCAF was only significantly retained by the GST-p73 fusion protein F2 that contains the central p73 region (aa 260-430), but not by the other constructs (Fig. 2B). PCAF did not bind to the GST protein alone (Fig. 2B, lane 6). The GST and GST-p73 fusion proteins retained in glutathione-Sepharose 4B beads in the assay were detected in Western blot analysis using antibody to GST (see lower part of Fig. 2B). To confirm these results, we incubated anti-Flag antibody, purified Flag-PCAF with protein G agarose in the presence of one of the same set of GST-p73 fragments, and the precipitated GST fusion protein was revealed in

Western blot analysis with anti-GST antibody. As shown in Fig. 2C, PCAF-F2 interaction was confirmed. In addition, F4 also binds to PCAF in the IP assay (lane 3 in the lower part of Fig. 3C). Both F1 and F3 failed to bind to PCAF (data not shown). We conclude that PCAF binds to a region in p73 that spans OD and a part of DBD as well as a unique C-terminal fragment present only in p73α.

PCAF acetylates p73. PCAF is the first identified mammalian acetylase and is a part of large complex that regulates transcription (48, 55, 60). PCAF binds to and acetylates p53 (31, 32, 46). As PCAF binds to p73, we wondered whether p73 is also a substrate of PCAF acetylation. To address this issue, various GST-p73 constructs described above were subjected to acetylation by purified PCAF in vitro. As seen in Fig. 3A, p73 fragment F4 was acetylated by PCAF (lane 7). Acetylation of p53 (as a positive control, lane 2) and PCAF self-acetylation (all lanes) were also detected. PCAF did not acetylate GST, even though significantly more GST was used as the substrate (compared lane 8 with other lanes in the Coomassie-stained gel on the left). On the same autoradiogram, acetylation of p73 fragments F1-3 was not apparent, although upon a much longer exposure, both F1 and F2 appeared to be acetylated (data not shown). Acetylation of F3 and GST was never detected under our assay conditions. The construct with GST fused to full-length p73α overlaps with the PCAF band on the gel (lane 3). To definitely demonstrate acetylation of full-length p73, we used a PCAF mutant that lacks the bromodomain (aa 719-832) as the acetylase. This mutant migrated faster than GST-p73α and also self-acetylated (Fig. 3B). It also acetylated GST-p73α (lane 2) and p53 (lane 3). Thus this mutant was fully active as an acetylase. Lane 1 shows PCAF only, which migrated at about the same position as GST-p73α (compare lanes 1 and 2), in agreement with results seen in panel A (lane 3).

Since F4 represents the major domain that is acetylated by PCAF, we wished to identify the lysine residue(s) that were modified by PCAF. There are only three lysine residues (Lys 620, 623, and 627) in F4. We mutated these lysines into arginine individually or in combination. The mutants fused with GST were expressed and purified. As expected, F4 was acetylated, but mutant K3R in which all three lysines were converted to arginines was not (Fig. 3C). Similarly, mutants K623R and K623-627R were not acetylated (Fig. 3D, lanes 2 and 5), whereas mutants K620-627R, K620R were still acetylated (lanes 3 and 4). These results indicate that lysine at position 623 was the specific PCAF acetylation site.

To verify if p73 is acetylated by PCAF in vivo, Saos-2 cells were cotransfected with plasmid pCX-Flag-PCAF with pcDNA3-p73α or pcDNA3-p73β. Thirty-six hours post-transfection, cells were treated with deacetylase inhibitor TSA for two hours. The extracts of transfected cells were subjected to IP with anti-HA antibody and the immunoprecipitates were analyzed using antiserum that specifically recognize acetylated-lysine residues. As shown in Fig. 3E, in vitro acetylated GST-p73 and PCAF (both migrated at the same position on the SDS gel, also see Fig. 3B) was easily detected by acetyl-specific antibody (lane 1). A species with the size of p73α was present in the anti-HA antibody immunoprecipitate from TSA-treated cells, as revealed in the Western blot analysis using antiserum against acetylated lysines (lane 3). Significantly, this band was absent in the precipitates from cells that were transfected with the same set of plasmids but were not treated with TSA (lane 2). No band with the molecular weight similar to p73β was detected in pcDNA3-HA-p73β transfected cells with or without TSA treatment. Curiously, a species about the size of PCAF was detected in all lanes, although the identity of this band is unknown. Both HA-p73α and HA-p73β were expressed in the transfected cells, as shown in the lower part of Fig. 3E. Thus, p73α is also acetylated in vivo.

PCAF stimulates p73-dependent transcription. PCAF is a transcription coactivator that enhances the activity of numerous activators (48, 51). To determine the functional relevance of the PCAF-p73 interaction and p73 acetylation by PCAF, we examined whether PCAF affects p73-mediated transactivation. A p53-responsive luciferase reporter PG13-Luc (8) was transfected along with selected plasmids into p53-deficient Saos-2 cells. As shown in Fig. 4A, transfection of PCAF stimulated reporter activity slightly, while p73α had little effects on PG13-Luc reporter whether or not PCAF was cotransfected. Interestingly, p73β transfection significantly stimulated the reporter activity, and cotransfection of PCAF dramatically enhanced the reporter activity. These data indicate that PCAF is a coactivator of p73β. Consistent with previous reports, our data also indicate that p73β may be a more potent transactivator than p73α. To examine the requirement of PCAF HAT activity for p73β-mediated transactivation, two HAT-deficient mutants of PCAF, PCAFΔHAT1 (Δ579-608) and PCAFΔHAT1 (Δ608-623), were used for cotransfection. Both mutants did not detectablely affect p73β-mediated transactivation (Fig. 4B). These observations could not be due to the lower expression of the mutants, as the protein levels of these mutants were comparable to that of wild-type PCAF in transfected cells (Fig. 4B). Thus, the HAT function of PCAF is required for p73β-mediated transactivation.

To further test the effects of PCAF on p53-responsive genes, we used the pWAF1-Luc reporter, which contains p21/cip1/WAF1 gene promoter. Consistent with published results (6, 67), both p73α and β can stimulate pWAF1-Luc reporter (Fig. 4C). Not surprisingly, transfection of PCAF alone significantly enhanced the reporter activity, as it was shown previously that PCAF can activate MyoD-dependent p21 transcription in myoblasts (43, 47). Collectively, these

data demonstrate that PCAF is a coactivator of p73-mediated transactivation and such coactivation requires PCAF HAT activity.

PCAF stimulates p73-dependent apoptosis in vivo. Overexpression of p73 can lead to cell cycle arrest or apoptosis (22, 24, 30, 68). To assess whether PCAF modulates these two biological efforts of p73, we examined the percentage of apoptotic cells in transiently transfected Saos-2 cells using various combinations of plasmids. Elevated apoptosis in p73α and β-transfected cells was found relative to control transfection, and PCAF can further increase apoptosis in these experiments (Fig. 5A). We then performed colony formation assays in p53-deficent small-cell lung carcinoma H1299 cells. The dose of neomycin-resistant gene and the transfection conditions were kept the same in all transfections. Thus, the number of G418-resistant colonies is a reflection of growth arrest and apoptosis exerted by the transfected plasmids in each transfection. PCAF moderately reduced the number of colonies, whereas the HAT-deficient mutants (PCAFΔHAT1 and 2) had virtually no effects on colony formation (Fig. 5B and C). p73α transfection reduced the colony number to about a half of the control, and PCAF cotransfection further decreased the colony number to ~20% of the control transfection. Introduction of p73β-expression plasmid significantly reduced the colony number (~40% of the control), and PCAF cotransfection led to further reduction. In addition, whereas HAT-deficient mutants moderately enhanced p73α-mediated reduction in colony formation, these mutants did not influence p73β-mediated effects, suggesting that the HAT activity of PCAF may be required for p73 especially p73β-mediated efforts on colony formation. These results support the notion that both p73α and β play a role in cell growth regulation, fully consistent with previous reports (24, 30, 59). Further, our data support a functional interaction between PCAF and both p73α and

β in cell cycle control and apoptosis, likely through their combined efforts in activation of genes involved in these processes.

Acetylation of lysine 623 by PCAF is important for p73α-mediated growth control. Having determined that PCAF acetylates K623 of p73α, we then examined potential consequence of this acetylation for p73α function. Three p73α mutants were tested for their effects on p73-mediated transactivation of reporter gene in Saos-2 cells and on colony formation in H1299 cells. Mutant p73αK3R has arginine in positions 620, 623 and 627, while p73αK620R and p73αK623R carry arginine in positions 620 and 623 respectively. As shown in Fig. 6, mutants that carry mutation K623R all had reduced efforts on transactivating pWAF1-Luc reporter (Fig. 6A) and on colony formation (Fig. 6B), while mutation K620R had virtually no effects in both assays. All three mutants as well as p73α were expressed in transfected cells, as illustrated in Fig. 6C. Thus, acetylation at 623 of p73α may be important for its function in regulating cell growth.

Discussion

We demonstrated here that PCAF is a p73 acetylase and transcriptional coactivator. PCAF is a coactivator that enhances the activity of numerous other activators and proteins involved in transcription, including class II transactivator (CIITA) (15, 49), MyoD (40, 47), E2F1 (35), TAL1/SCL (17), RXR-RAR nuclear receptors (4), E1A (65), and TAF (38), and architectural protein HMGI(Y) (37). PCAF can function alone or together with p300/CBP coactivators through their interaction depending on targets. Interestingly, the HAT activity of p300/CBP and PCAF is often differentially used in transcription activation. For example, although both p300/CBP and PCAF are required cofactors for MyoD, only the HAT activity of PCAF but not

that of p300/CBP is necessary for MyoD activity and muscle differentiation (43). Moreover, PCAF acetylates MyoD, which augments the MyoD's DNA binding activity (47). Similarly the HAT activity of PCAF rather than that of p300/CPB is required for transcription mediated by the retinoic acid receptors (RARs) (27). In contrast, CREB- and STAT-1-mediated transactivation relies on the HAT activity of p300/CBP (27). Nonetheless, both PCAF and p300/CBP acetylate histones, which are thought to be required for general transcription (5, 52). Thus, different activators could in principle recruit either p300/CBP or PCAF for targeted nucleosomal acetylation of specific genes. In addition, acetylation of the architectural protein HMGI(Y) (37) by both PCAF and p300/CBP plays distinct role in coordination of transcriptional switch of the interferon-β gene expression (37).

It was shown that p300/CBP is a coactivator for p73-mediated transactivation. The TAD of p73 binds to the CH1 domain of p300/CBP and this interaction is necessary for coactivation (64). Interestingly, the p300/CBP HAT activity does not appear to be required for stimulating p73-medaited transactivation (63). Here we show that PCAF also functions as a potent coactivator of p73. Furthermore, the HAT domain of PCAF is necessary for this activity. Thus, like MyoD and RARs, p73 uses both PCAF and p300/CBP as coactivators and only the HAT activity of PCAF is required for p73-mediated transactivation. It remains to be determined whether p73 recruits p300/CBP and PCAF concomitantly for activating its target genes or differentially employs these coactivators in a gene-specific manner.

p300/CBP is the coactivator for p53-mediated transactivation (3, 14). Like p73, p53's TAD binds to p300/CBP, although there are two mapped binding sites in p300/CBP for this p53 domain: the CH3 domain and a region near the C-terminus (11). Further, a region with the p53 DBD binds to the CH1 domain of p300/CBP (11). The interaction between the p53's TAD and

p300/CBP is believed to be required for activating p53-mediated transactivation (11). Both p300/CBP and PCAF acetylates p53 (13, 31, 32). While p300/CBP as a coactivator of p53 transactivation is clearly documented, whether PCAF is also a p53 coactivator is unclear. Mutation of PCAF acetylation site in p53 did not significantly affect p53's transactivation potential (31). Further, while p300 can clearly activate p53-mediated transcription in an in vitro transcription system, PCAF had little effect (9). In a transient transfection assay, PCAF did not stimulate p53-mediated transcription of reporter gene (S. Anderson and D. Liao, unpublished results). Thus, acetylation by PCAF may not play a critical role in stimulating p53-dependent transcription under normal conditions, it might have other consequence (for p53 function) instead. Our unpublished data indicated that acetylation of p53 by PCAF may regulate p53 nuclear retention (S. Anderson and D. Liao, unpublished results).

The fact that p53 uses only p300/CBP and p73 recruits both p300/CBP and PCAF as coactivators may have functional consequence. Although the DBD is the most conserved domain between p53 and p73, and they bind to the same consensus sequence, their target genes may not overlap entirely. For example, only six out of 36 p53-induced genes were significantly induced by p73α (61). In contrast both p73α and β are much more potent in activating 14-3-3σ than p53 (67). An important issue in understanding p73 biology is to identify specific target genes for p73. The knowledge that PCAF is a coactivator of p73-dependent transactivation may facilitate this task.

Consistent with previous results that expression of both p73α and β can induce p21/cip1/WAF1 gene (24, 67), we showed that they also activate p21 promoter in our reporter gene assays (Fig. 4C). Interestingly p73α was completely inert in activating artificial reporter PG13-Luc (Fig. 4A and B). Although unlikely, the simplest explanation is that p73α does not

bind to PG13-Luc. Alternatively, p73α-mediated transactivation requires interactions with other factors that can associate with p21 promoter but not with the PG13 synthetic promoter. Whatever the reason may be, our data point out the subtle difference between p73 isforms in transactivation. One implication is that p73 isforms may regulate the expression of different target genes or the same set of genes under different conditions.

The biological effects of acetylation of transcription activators are not entirely clear. Acetylation can enhance protein-protein and/or protein-DNA interaction, nuclear localization as well as protein stability (28). Acetylation of p53 can enhance its ability to bind to DNA (13, 46) as well as its stability (20). Both effects can be important for p53 biochemical function. Interestingly, although acetylated p53 binds to a short stretch of naked DNA better than non-acetylated p53, acetylation had no obviously effect on p53's ability to bind to chromatin and to activate transcription from nucleosomal template in vitro (9). Thus, acetylation of p53 may not play a critical role in stimulating p53 DNA binding. Nonetheless, p53 acetylation play an important physiological role in regulating p53 function, as p53 acetylation is induced in response to a variety of stress signals (20, 46) and both viral and cellular oncoproteins inhibit p53 acetylation (20, 26, 32).

We show here that p73α is significantly acetylated in vitro and in vivo, and that p73β is not acetylated (Fig. 3). Although both fragments F1 and F2 were acetylated to some extent in vitro, we were unable to detect acetylated p73β in vivo (Fig. 3E). However, the HAT domain of PCAF is clearly required for stimulating p73β-mediated transactivation (Fig. 4B). This is not unexpected, as p53 acetylation per se is not necessary for p53-mediated transactivation; rather, DNA-bound p53 recruits p300/CBP, leading to targeted nucleosomal acetylation and transcription activation (9). p73β may act in the same way by recruiting PCAF to specific

promoters for targeted acetylation and transcription activation. Nonetheless, we showed that acetylation of p73α may affect its transactivation potential, as mutants that cannot be acetylated at K623 failed to activate pWAF-Luc reporter and such mutants were also less potent in reducing colony number in colony formation assay (Fig. 6). Acetylation of p73α can potentially affect its stability, subnulcear localization and interaction with other proteins, all of which can influence its transactivation potential.

The PCAF acetylation site of p73 lies within the unique sequence only found in p73α and ΔN-p73α. Thus, acetylation might also affect ΔN-p73α function. It is thought that different isoforms of p73 may have distinct roles during development (59). Thus, acetylation of specific isoforms can in principle come into play in regulating gene expression and apoptosis in development. The full functional ramification of p73 acetylation remains a question for future investigation.

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Liu et al., Fig. 1

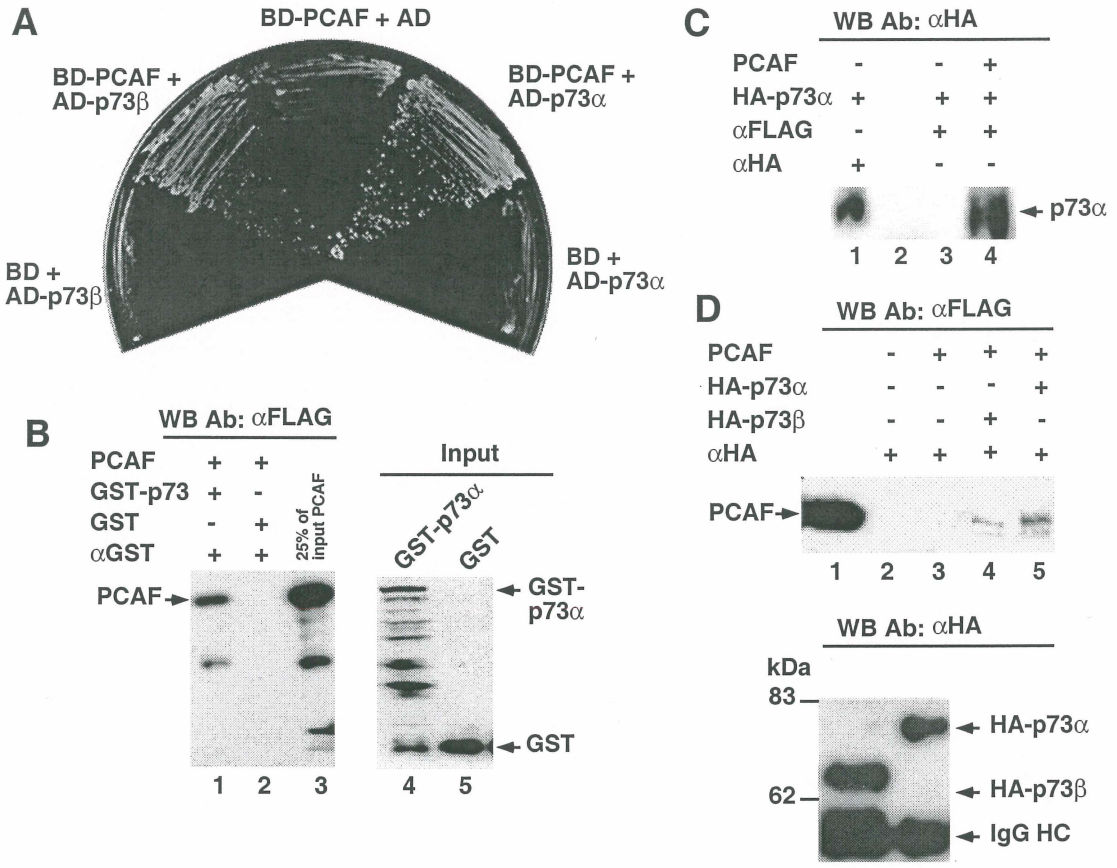

FIG. 1. p73 binds to PCAF in vitro and in vivo. (A) p73α and β interact with PCAF in yeast two hybrid assays. PCAF was fused to Gal4-BD and p73α and β were fused to Gal4-AD. Various combinations of plasmids as indicated were introduced into yeast and its growth in medium lacking histidine but in the presence of 3-AT was scored. (B) p73α binds to PCAF in vitro. Purified GST-p73α and Flag-tagged PCAF were incubated with anti-GST antibody and protein G agarose and washed extensively. Proteins that remained in the beads were separated in SDS-PAGE and detected with anti-Flag antibody in WB. The input PCAF (lane 3), GST-p73α and GST (lanes 4 and 5) are shown. (C and D) PCAF-p73 interaction in vivo. Plasmids expressing HA-p73α and β and Flag-PCAF were transfected into Saos-2 (C) or HeLa (D) cells and the extracts of transfected cells were subjected to IP and the immunoprecipitates were analyzed in WB with indicated antibodies.

Liu et al., Fig. 2

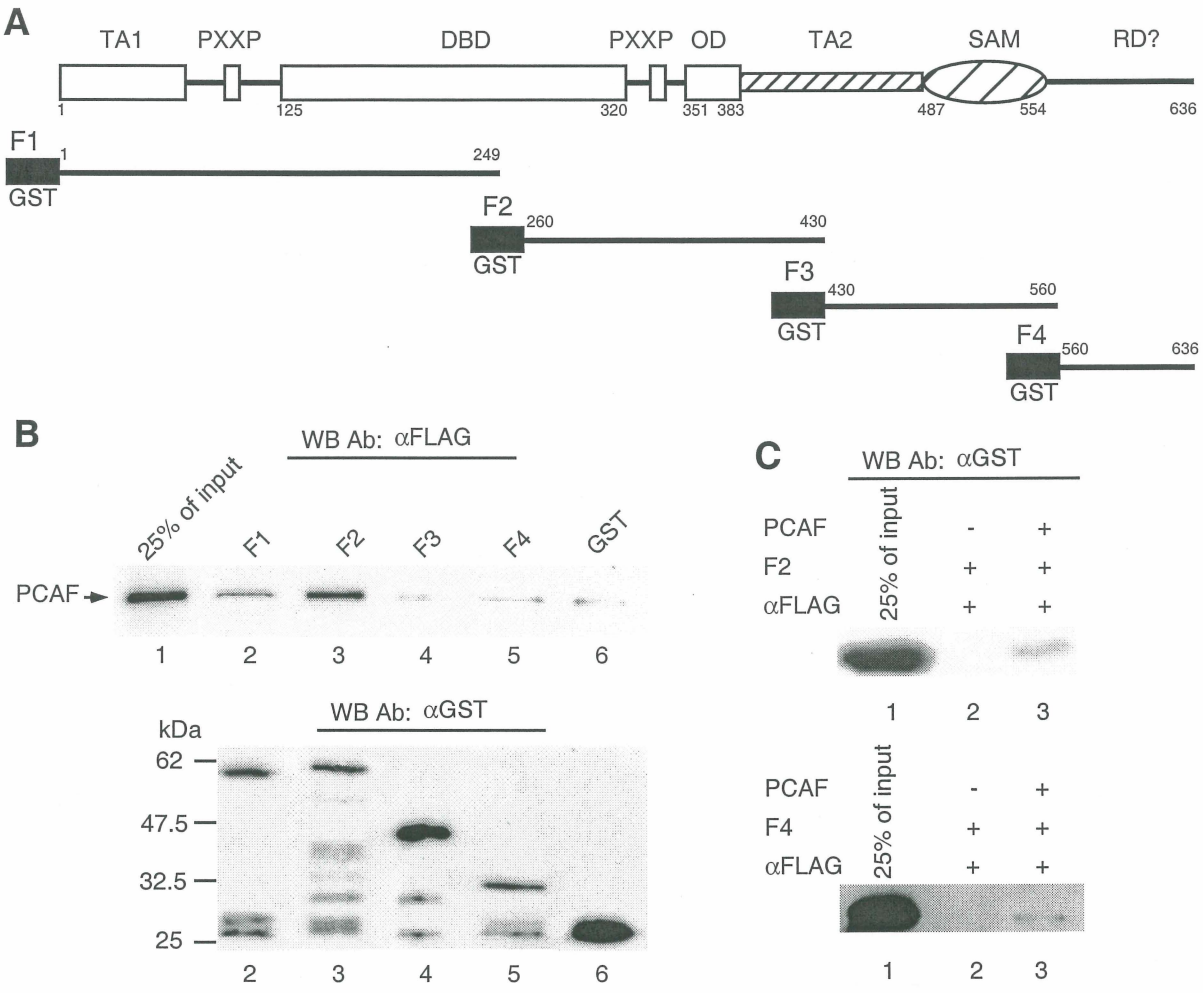

FIG. 2. PCAF interacts with the central domain of p73. (A) Schematic representation of p73α structural domains. TAD: transactivation domain, PXXP: praline-rich motif, DBD: sequence-specific DNA-binding domain; OD: oligomerization domain, SAM: sterile-α domain, RD: regulatory domain. The numbers denote the amino acid position. The constructs that contain the fusion of GST and p73α fragments that span its entire length are depicted. (B) GST pull-down assay indicating that PCAF binds to p73α F2 fragment. Purified Flag-PCAF and GST-p73 fusion proteins (F1-F4) were incubated with glutathione Sepharose 4B beads and the mixtures were washed extensively. The proteins that remained in the beads were subjected to SDS-PAGE and WB using indicated antibodies. The upper panel shows the GST-p73 fusion proteins that associated with Flag-PCAF, and the lower panel shows beads-bound GST or its fusion with p73α fragments. (C) IP from purified protein mixture. F2 and F4 were incubated with Flag-PCAF and subjected to IP and WB using indicate antibodies.

Liu et al., Fig. 3

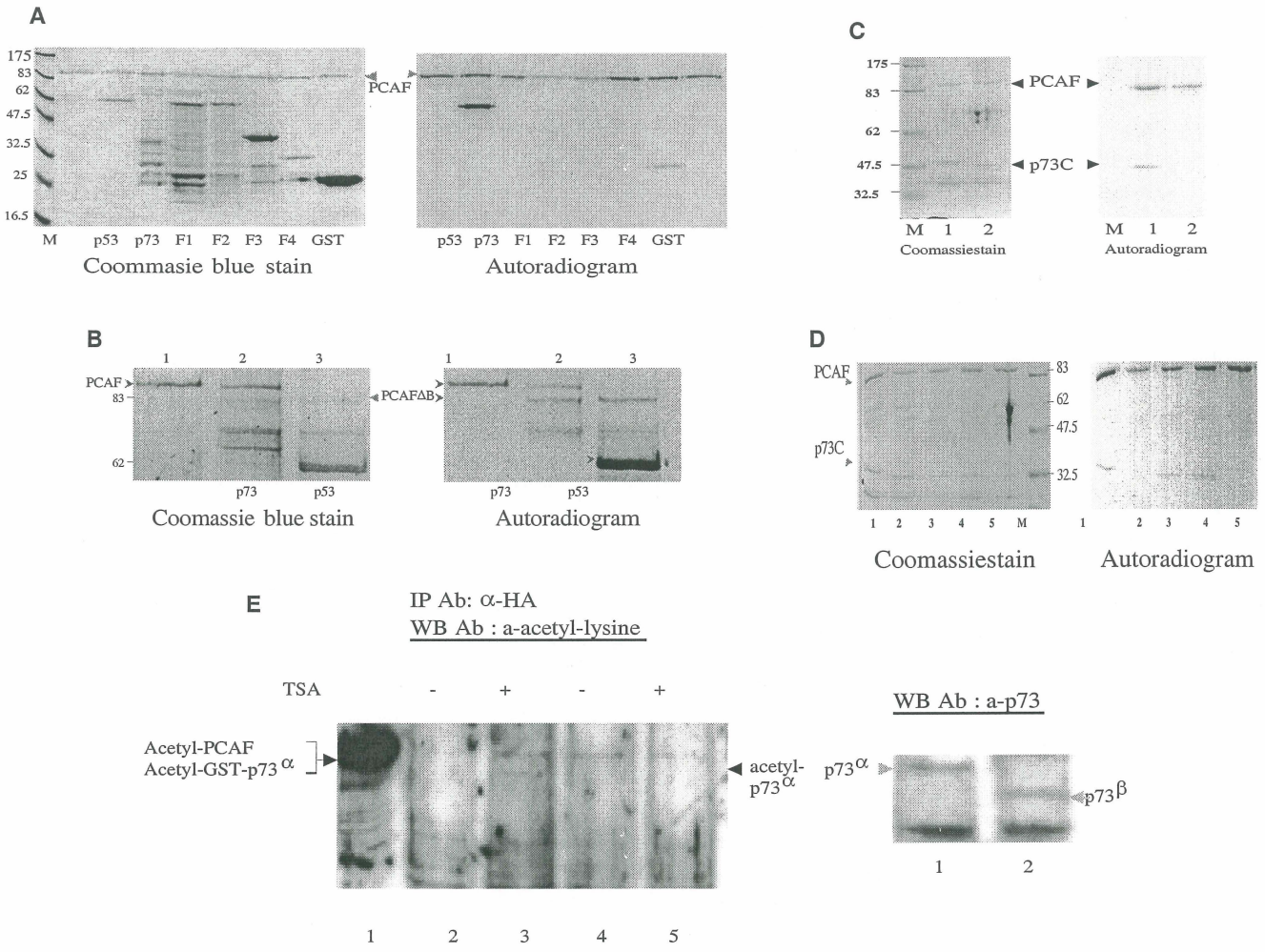

FIG. 3. Acetylation of p73 by PCAF in vitro and in vivo. (A) p73α C-terminal domain is acetylated by PCAF. Fusion protein between GST and full-lengthp73α (lane 3), F1 (aa 1-249, lane 4), F2 (aa 260-430, lane 5), F3 (aa 430-560, lane 6) and F4 (aa 560-636, lane 7) was incubated with PCAF for in vitro acetylation. Controls include PCAF alone (lane 1), PCAF + p53 (lane 2) and PCAF + GST (lane 8). The right panel is the autoradiogram of the Coomassie blue-stained SDS-polyacrylamide gel shown on the left. M is the protein size ladder and the molecular weight in kDa is indicated. (B) Acetylation of p73α by PCAF mutant lacking the bromodomain (PCAFΔB). The aa 719-832 of PCAF was deleted in this mutant, which was incubated with either GST-p73α (lane 2) or p53 (lane 3); lane 1 shows PCAF only. Both p53 and GST-p73α were acetylated by this mutant. Both Coomassie blue-stained gel (left) and autoradiogram (right) were shown. (C and D) Lysine 623 of p73α is the PCAF acetylation site. Lysines at 620, 623 and 627 within F4 (aa 560-636) were mutated individually or in combination. The mutants were fused with GST and purified from *E. coli* and used as substrates for in vitro acetylation with PCAF. Lysine-arginines conversions in these mutants are: K3R, all three lysines; K623R, Lys 623; K620-627R, Lys 620 and 627; K620R, Lys 620; K623-627R, Lys 623 and 627. (E) acetylation of p73 in vivo. pCX-Flag-PCAF (2 μg) along with either pcDNA3-HA-p73α (lanes 2 and 3) or pcDNA3-HA-p73β were transfected into Saos-2 cells. After growth for 36 h, cells were treated with TSA (5 μM, +) or untreated (-) for 2 h and then harvested. The extracts were subjected to IP with anti-HA antibody and the immunoprecipitates were analyzed with antiserum against acetyl-lysines in WB. The in vitro acetylation reaction mixture using GST-p73α and PCAF was loaded in lane 1. The expression of HA-p73a and b in the transfected Saos-2 was verified by IP with antibody to HA tag and WB with anti-p73 antibody (H-79, Santa Cruz Biotechnology).

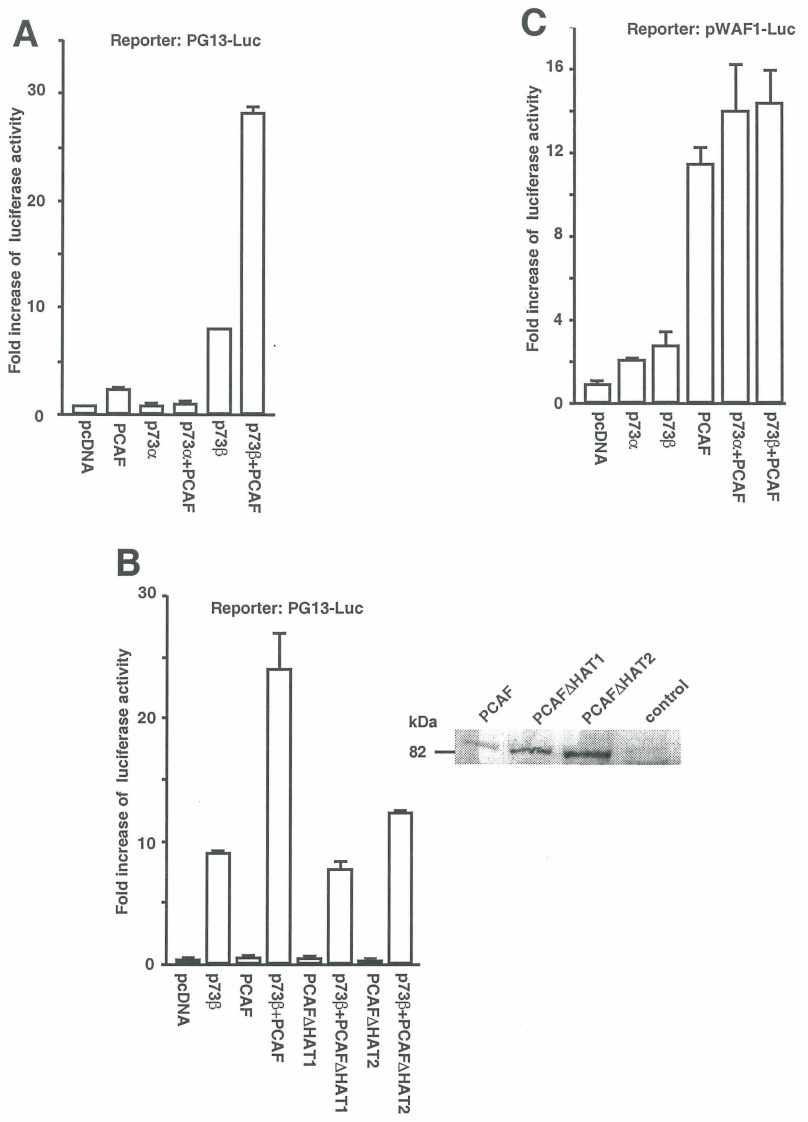

FIG. 4. PCAF is a coactivator of p73-mediated transactivation. (A) Firefly luciferase reporter PG13-Luc containing multiple copies of consensus p53-binding sites along with internal control Renilla luciferase reporter was transfected with empty vector pcDNA3.1 (pcDNA), or pCX-Flag-PCAF (PCAF), pcDNA3-HA-p73α (p73α), pcDNA3-HA-p73β (p73β) into Saos-2 cells and dual luciferase assays were carried out. The firefly luciferase activity was normalized against Renilla luciferase activity, and the luciferase activity relative to control (pcDNA) was plotted. (B) The HAT domain of PCAF is required for stimulating p73β-mediated transactivation. PCAF HAT mutants (ΔHAT1, Δaa 579-608 and ΔHAT2, Δaa 608-623) were used in lieu of PCAF in the reporter assays. The reporters and other plasmids used for transfection were as in (A). The expression of PCAF and its HAT mutants in the transfected cells was verified in WB using anti-Flag antibody. (C) PCAF as coactivator of p73α and β for p21/cip1/WAF1 promoter. The assays were as in (A) except that the firefly luciferase reporter was pWAF1-Luc that contains the p21/cip1/WAF1 gene promoter.

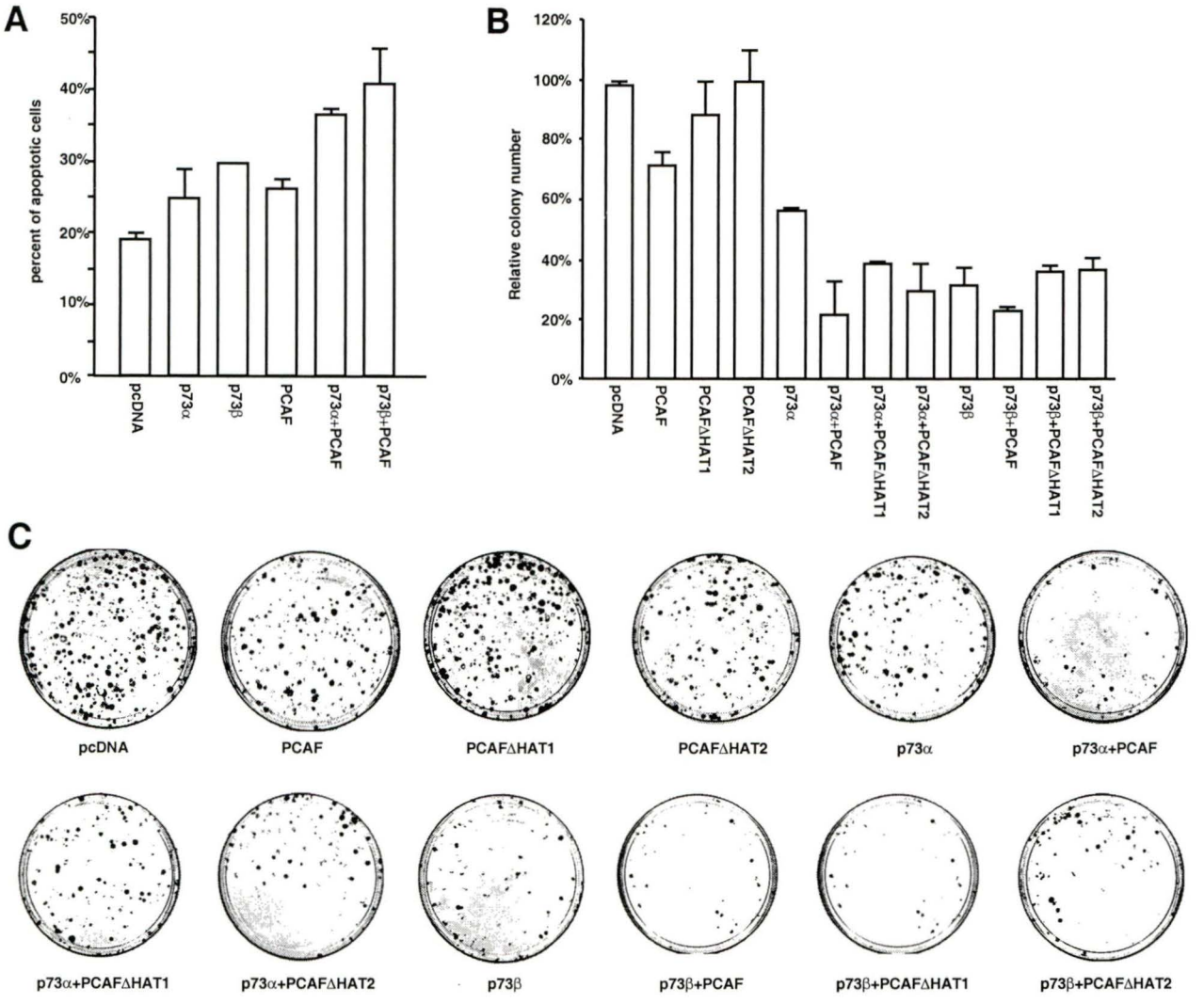

FIG. 5. PCAF enhances the negative cell-growth regulation of p73α and β. (A) PCAF affects p73α- and β-i apoptosis. Various expression plasmids as shown in Fig. 4 were transfected into Saos-2 cells along with pEC The morphology of transfected cells exhibiting green fluorescence was examined. Apoptotic cells were iden by their rounded and shrunken morphology in contrast to the spread-out appearance of non-apoptotic cells, on a blind basis, and presented as a percentage of the total population of fluorescent cells. (B and C) PCAF es p73α- and β-mediated reduction of colony numbers in colony formation assay. H1299 cells were transfec using various expression plasmids and the transfected cells were grown in RPMI medium containing 0.4 mg G418. Drug-resistant colonies were stained with Giemsa and counted. Panel B shows a graph of colony nun from transfections with various expression plasmids (see Fig. 4) relative to transfection with pcDNA3.1 emp vector. The dose of neomycin-resistant gene and total amount of DNA in each transfection was exactly the s More than three independent transfections were performed with essentially identical results. Representative of Giemsa-stained plates from different transfections are shown in (C).

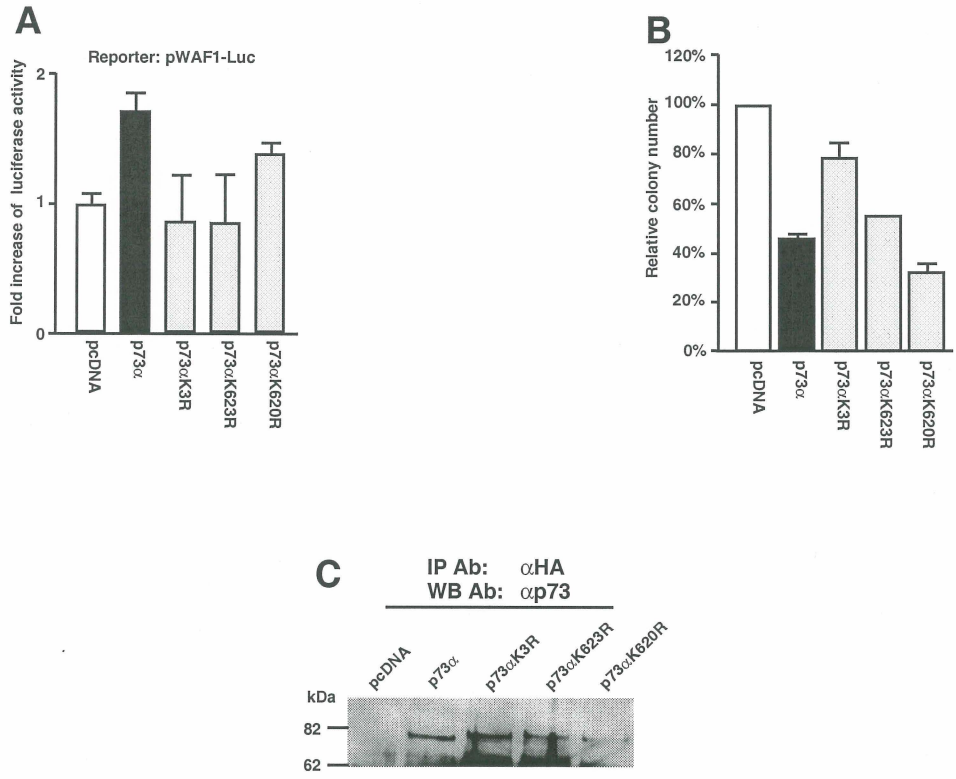

FIG. 6. Acetylation of lysine 623 by PCAF is important for p73α-mediated cell-growth regulation. (A) Mutation at Lys 623 reduces p73α-dependent transactivation. pWAF1-Luc and Renilla-Luc reporters were transfected into Saos-2 cells with pcDNA3.1 (pcDNA) or with pcDNA3.1-HA-p73α (p73α), pcDNA3.1-HA-p73αK3R (p73αK3R, in which K620, 623 and 627 were changed to R), pcDNA-HA-p73αK623R (p73αK623R, K623 was converted to R), pcDNA-HA-p73αK620R (p73αK620R, K620 was mutated to R), and the dual luciferase assays were conducted as in Fig. 4. (B) Colony formation assays using p73α or its mutants. The assays were performed as in Fig. 5. (C) Expression of p73α and its mutants in transfected Saos-2 cells. The extracts of transfected cells using indicated expression plasmids were subjected to IP with antibody to HA tag and the immunoprecipitates were analyzed using antibody to p73 (H-79) in WB.

Chapter 4 Sub-project 3

Identification of the p53 and AdE1B 55K Binding Sites on PCAF

Introduction

Adenovirus E1B 55-kDa oncoprotein (E1B) binds to and inactivates human tumor suppressor p53 (Arrowsmith 1999). Our previous study indicated that one way for E1B to inactivate p53 is to inhibit its acetylation by PCAF (Liu et al., 2000). We showed that E1B binds to p53 and PCAF, its interaction with p53 and PCAF may dissociate the weak substrate-enzyme interaction between p53 and PCAF and hence inhibit p53 acetylation. To further study the mechanistic detail of how E1B inhibits p53 acetylation by PCAF, we identified the p53 and E1B binding sites on PCAF.

We found that p53 binds to the central domain of PCAF and E1B binds to the C-terminal bromodomain of PCAF. E1B can inhibit p53 acetylation by the bromodomain-deleted PCAF (PCAFΔB, aa 719-832 deleted). Since PCAFΔB cannot bind to E1B, this result confirms that the E1B-p53 interaction plays an important role in inhibiting p53 acetylation by PCAF. We also studied if E1B affects p73 function as regulated by PCAF. We found that E1B not only failed to inactivate p73α and p73β dependent transcription, which is consistent to published data (Higashino et al., 1998; Steegenga et al., 1999), but also failed to prevent PCAF from stimulating p73-dependent transcription. Furthermore, E1B does not inhibit p73C acetylation by PCAF. Therefore, we confirmed that the adenovirus E1B 55K protein distinctly regulates p53 and its family member, p73.

Materials and Methods

Protein preparation. The baculoviruses producing PCAF, PCAF N (aa 1-529), PCAFΔN (aa 62-464 deleted) and PCAFΔB (aa 719-832 deleted) and some purified proteins produced by these viruses were kindly provided by Dr. Xiangjiao Yang (Oncology group, McGill University). The PCAF, E1B and p53 proteins were prepared in the same way as described in chapter 2. The pET14B-Bromodomain plasmid containing His-tagged Bromodomain of PCAF (aa 719-832) was a kind gift from Dr. Mingming Zhou (Mount Sinai School of Medicine, USA). The His-bromodomain was expressed in *E. coli* and purified with Ni-NTA (nickel-nitrilotriacetic acid) agarose (Qiagen).

Coimmunoprecipitation (IP) and Western blot (WB) analysis. In each IP experiment, approximately 0.5 to 1 μg of each purified protein or 20 μg of cell lysate were incubated with an appropriate antibody in buffer B (same as in chapter 2) for at least 1 h with rotation at 4°C. The amount of antibody used per IP assay is different depending on specific antibodies; the typical amount is 1 μg. Protein G agarose beads (30 μl; Roche Molecular Biochemicals) were added into the protein-antibody mixture and incubated at 4°C for at least 1 h with rotation. The beads were collected by centrifugation and washed 3 times with buffer B. The precipitated proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in 25 mM Tris base-190 mM glycine at 50 V

for 2 h at 4°C. The coprecipitated proteins were detected using an appropriate antibody with the enhanced chemiluminescence (ECL, Amersham-Pharmacia Biotech) kit.

Acetyltransferase assay. The human PCAF or PCAFΔB protein was used as acetylase. Purified p53 was subjected to acetylation by PCAFΔB or PCAF, p73 C-terminus (aa 558-636) was subjected to acetylation by PCAF. E1B proteins were added in some of the reactions as indicated in Fig. 8 and 9. Protein samples were incubated at 30°C for 30 min in a total volume of 20 μl in acetylation buffer (same as in chapter 2) with 90 pmol of 1-^{14}C-acetylcoenzyme A (55 mCi/mmol; Amersham Pharmacia Biotech). The reaction mixtures were then analyzed by SDS-PAGE, and the gels were stained with Coomassie brilliant blue, dried, and were subjected to autoradiography (3 to 6 days).

Luciferase assay. SaoS-2 cells (50% confluent) were transfected with the PG13-firefly luciferase (Luc) reporter which contains multiple copies of the p53-binding sequence, or pWAF1-Luc, which contains a fragment from the p21/CIP1/WAF1 gene promoter, together with a CMV promoter-driven Renilla luciferase (RLuc) reporter plasmid and a combination of different plasmids as indicated in various figures using Superfect reagent. Irrelevant plasmids were added so that the total amount of DNA transfected was kept the same in each transfection. Cells were harvested for luciferase assays 18 h post-transfection using dual luciferase assay regents (Promega) according to the protocol supplied by the manufacturer. Firefly luciferase activity was normalized against RLuc activity.

Results

p53 binds to the central domain of PCAF.

In order to identify the p53 binding domain on PCAF, IP was performed with purified recombinant p53 and PCAF proteins. We performed IP by incubating murine anti-p53 antibody DO-1, protein G agarose and equal amounts of PCAF or PCAF mutants (Fig.6.). Precipitated proteins were analyzed by Western blot. As seen in Fig. 6, PCAF, PCAFΔB (F2) and PCAFΔN (F3) are coprecipitated with p53, but not the N-terminus of PCAF(F1) and bromodomain of PCAF (F4), indicating a direct interaction between p53 and PCAF central domain in vitro.

E1B binds to the Bromodomain of PCAF

Previous study of our lab (unpublished) has shown that in a yeast two-hybrid library assay (HeLa library) with Ad2E1B as a bait, E1B interacts with the bromodomain of the Bdf2 protein. Since the bromodomain is found in nearly all histone acetyltransferase including PCAF and is usually involved in protein-protein interaction, and E1B-PCAF interaction is detected both in vivo and in vitro (chapter 2). Here we test whether the bromodomain of PCAF is the E1B binding domain. As shown in Fig. 7, IP was performed by incubating protein G agarose and anti E1B antibodies with the purified E1B proteins and the His-tagged bromodomain of PCAF. Precipitated proteins were analyzed by Western blot. As seen in Fig 7A, His-bromodomain is coprecipitated with

both Ad2 and Ad12E1B. In Fig. 7B and 7C, we show that E1B failed to coprecipitate with the bromodomain-deleted PCAF mutant, thus indicating a direct interaction between E1B and the PCAF bromodomain in vitro.

E1B inhibits p53 acetylation by PCAFΔB

We have proposed in chapter 2 that E1B may inhibit p53 acetylation by PCAF by shifting the protein-protein interaction equilibrium from p53-PCAF to E1B-p53 and E1B-PCAF. Since a mutant E1B that fails to bind to PCAF but still binds to p53 can dissociate p53-PCAF interaction in a reverse yeast two-hybrid assay (chapter 2, Fig. 9), we suspected that the E1B-p53 interaction might be sufficient to inhibit p53 acetylation by PCAF. To test this possibility, we performed the in vitro acetylation assay by incubating PCAFΔB with p53 and ^{14}C-acetyl-CoA. E1B was added in some of the reactions as indicated in Fig 8. We showed that PCAFΔB acetylates p53 (lane 3), and E1B proteins inhibit p53 acetylation by PCAFΔB (lanes 1 and 2). Since PCAFΔB does not bind to E1B, this result confirms that the E1B-p53 interaction plays an important role in inhibiting p53 acetylation by PCAF.

E1B failed to inhibit p73 function regulated by PCAF

A previous study has shown that adenovirus E1B 55K oncoprotein failed to inactivate transactivation function of p73 (Steegenga et al., 1999). To conform this and to further study if E1B affects the function of p73 when stimulated by PCAF, we performed the luciferase reporter gene assay. A p53-responsive luciferase reporter PG13-Luc or pWAF1-Luc was transfected into p53-deficient Saos-2 cells along with selected plasmids

as indicated in Fig 9. We show that E1B failed to inhibit both p73 and PCAF stimulated transcription (Fig 9), although in the same reporter system, E1B significantly inhibits p53-dependent transcription (data not shown). Furthermore, E1B failed to prevent PCAF from stimulating p73 transcriptional function (Fig. 9). The result confirmed that adenovirus may not utilize its E1B 55-kDa oncoprotein to inactivate p73 (Higashino et al., 1998; Steegenga et al., 1999; Wienzek et al., 2000). Then we studied whether E1B can inhibit p73 acetylation by PCAF. In vitro acetylation assays were performed by incubating the p73 C-terminal fragment (aa 558-636) or p53 with PCAF and ^{14}C labeled acetyl-CoA in the presence or absence of the Ad12 E1B protein. We show that in the presence of E1B, while p53 acetylation is inhibited, p73C acetylation was not affected, suggesting that E1B does not inhibit p73 acetylation.

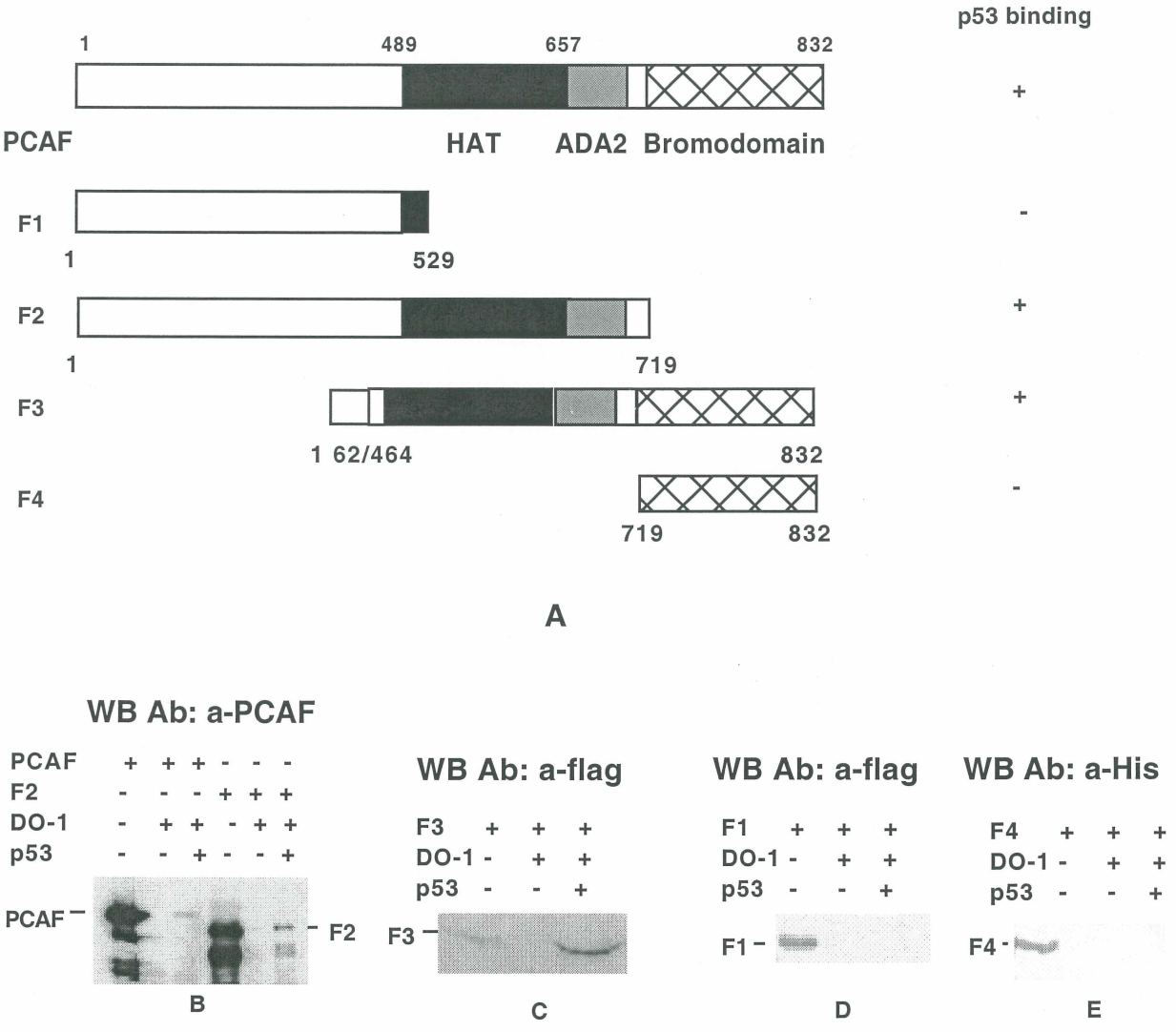

Fig. 6. p53 binds to the central domain of PCAF. (A) Schematic representation of PCAF structural domains. HAT: Histone acetylation domain, ADA2: binding domian of ADA2, a transcriptional adaptor. The constructs that contain the PCAF fragments that span its entire length are depicted. "-" represents "undetected", "+" represents "detected". (B,C,D) p53 binds to PCAF and PCAF F2, F3, but not F1. Purified p53 and Flag-tagged PCAF, F2 (in B), F3 (in C) and F1 (in D) were incubated with anti-p53 antibody DO-1 and protein G agarose and the mixtures were washed extensively. The proteins that remained in the beads were subjected to SDS-PAGE and WB using anti-PCAF antibody or anti-flag antibody as indicated. (E) p53 does not bind to PCAF F4. His-tagged PCAF F4 was incubated with DO-1, protein G agarose in presence and absence of p53. His-F4 cannot be detected from the precipitates in both cases.

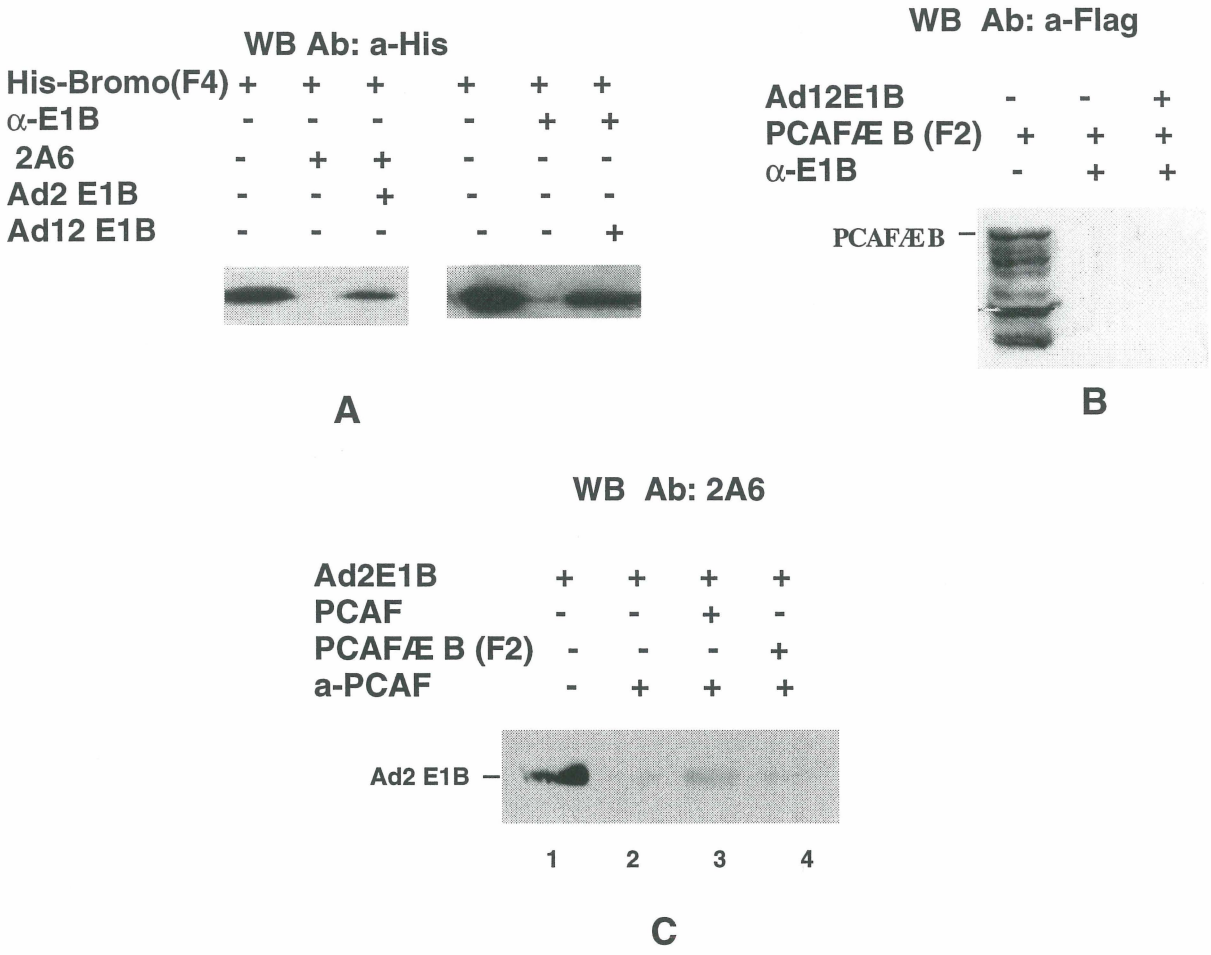

Fig. 7. E1B binds to the bromodomain of PCAF. (A) Both Ad2 and Ad12E1B coprecipitate with PCAF bromodomain. Purified His-PCAF bromodomain (F4) was inbubated with protein G agarose and anti-Ad 12E1B antibody α-E1B or anti-Ad2E1B antibody 2A6 in the presence or absence of purified E1B proteins. In the presence of E1B proteins, F4 was coprecipitated with both Ad2 and Ad12 E1B. (B)Ad12E1B does not interact with the bromodomain-deleted PCAF(F2). The insect cell (sf9) lysate containing PCAF F2 was incubated with protein G agarose and α-E1B in the presence or absence of purified Ad12E1B. F2 can not be detected in both cases. (C) Ad2 E1B does not bind to PCAF F2. Purified Ad2 E1B was incubated with protein G agarose, anti-PCAF antibody and PCAF or PCAF F2. While Ad2 E1B is coprecipitated by PCAF, it failed to be recoverd with PCAFÆB.

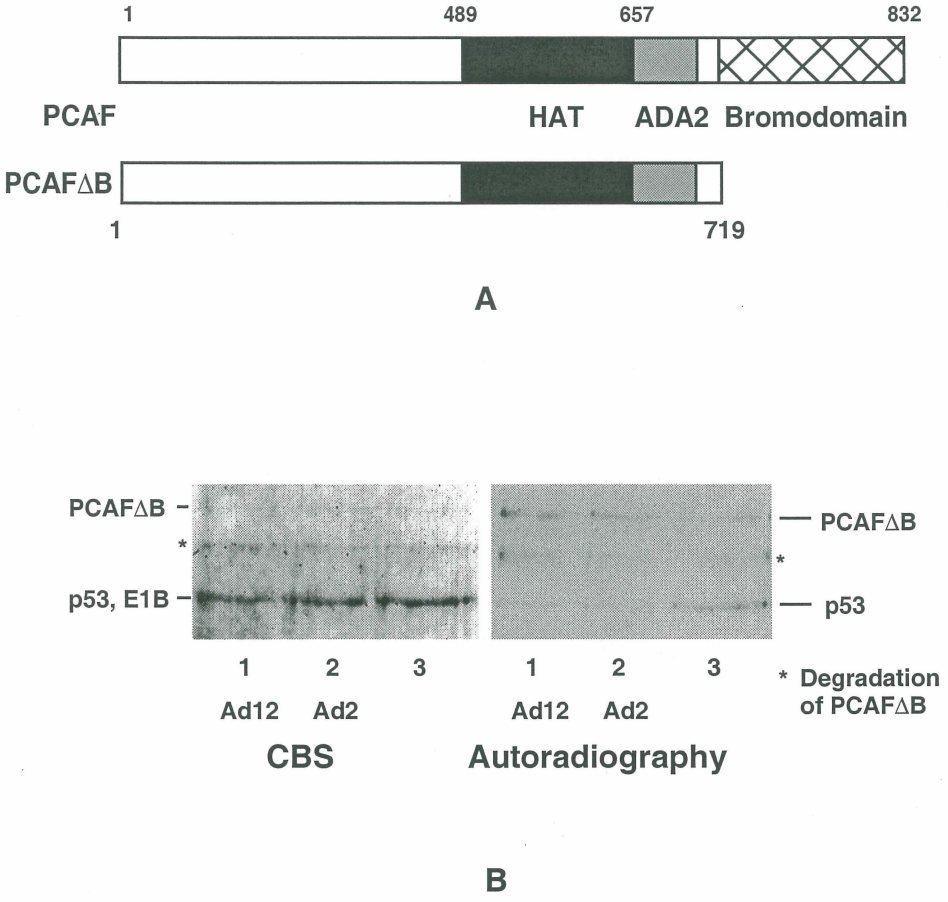

Fig. 8. E1B inhibits p53 acetylation by PCAFΔB. (A) Schematic representation of PCAF and bromodomain-deleted PCAF. (B) In vitro acetylation assay was performed by incubating purified p53 with purified PCAFΔB and ^{14}C labeled acetyl-CoA in the presense or absence of E1B proteins. In the presence of Ad2 E1B (lane 2) and Ad12 E1B (lane 1), p53 acetylation was specifically inhibited, the self-acetylation of PCAFΔB was not affected. The molecular weight of E1B and p53 are roughly the same, thus the two proteins migrate at the same position.

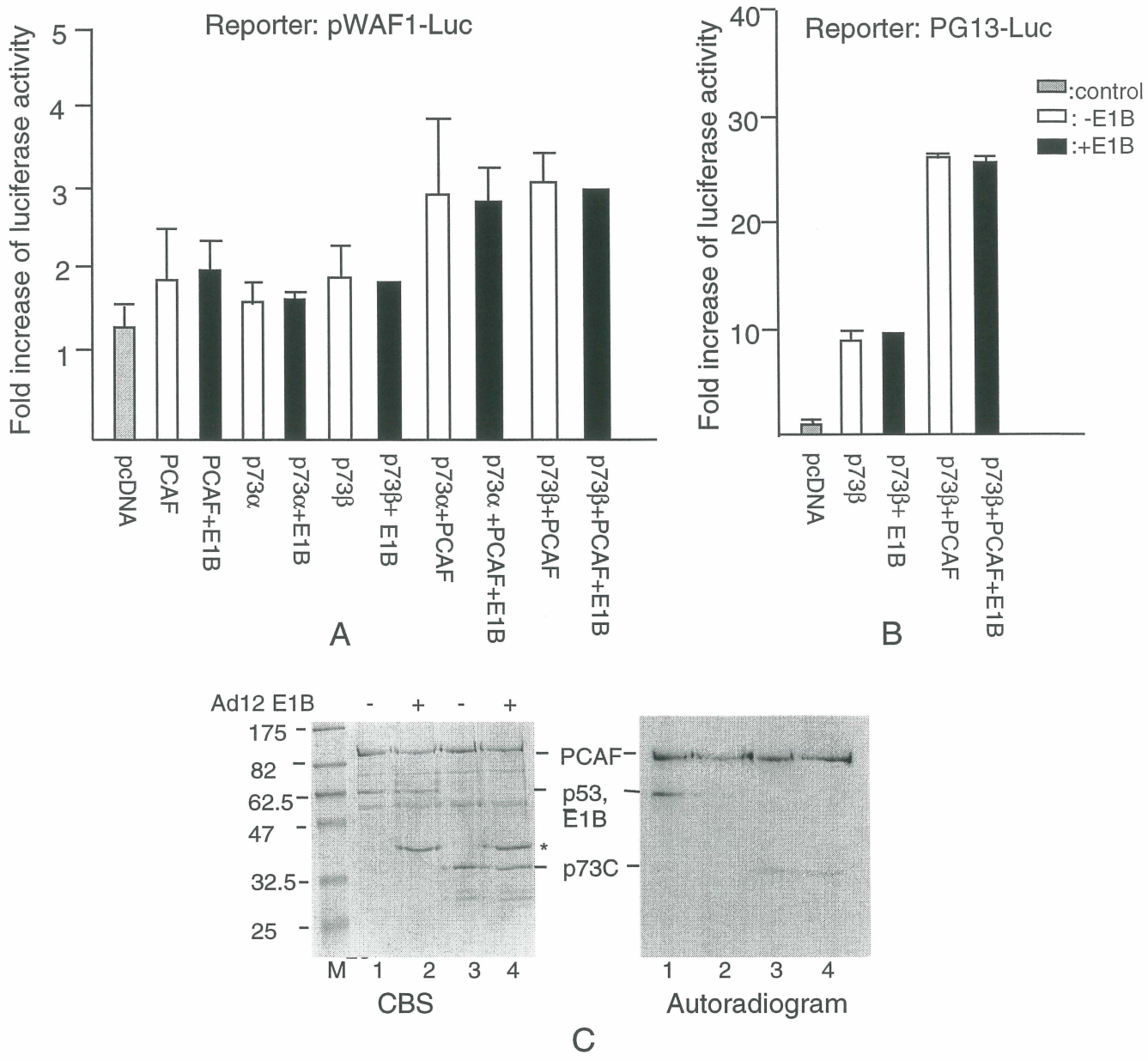

Fig. 9. E1B does not affect PCAF function on p73. (A) p21/cip1/WAF1-firefly luc reporter (1 ug) along with internal control Renilla luc reporter (1 ug) was transfected into Saos2 cells with empty vector pcDNA(100 ng) or pcDNA-HA-p73α (100 ng) , pcDNA-HA-p73β (100 ng) and plasmid pCX-Flag-PCAF (100 ng in # 2,3 of A and 1 ug in other columns) in the presence or absence of plasmid PCDL-SRa 296-Ad12E1B 55K(1ug). Dual luciferase assays were carried out. The firefly luciferase activity was normalized with Renilla luciferase activity, and the luciferase activity relative to control (pcDNA) was plotted. (B) Firefly luciferase reporter PG13-Luc was used for luciferase assays. Assays were performed the same way as indicated in (A). (C) PCAF, p53 (lane 1 , 2) or p73C (aa 560-636, lane 3, 4) were incubated with ^{14}C labeled Acetyl-CoA in the absence or presence of E1B. As seen in the autoradiogram, E1B did not affect PCAF autoacetylation and p73C acetylation but inhibit p53 acetylation. " * " represents a degradation band of E1B or a non-specific band from E1B purification.

Chapter 5 General Discussion and Conclusion

The tumor suppressor protein p53 has been called the "cellular gatekeeper" and a "guardian of the genome" due to its critical role in controlling cell cycle arrest and apoptosis (Lane, 1992; Levine, 1997). DNA damage, telomere erosion, aberrant proliferating signals, hypoxia, loss of adhesion or survival signals and viral infection all lead to activation of p53 (Douglas et al., 2001). In response to these signals, p53 can inhibit cell growth of damaged cells and allows for DNA repair or stimulate programmed cell death. Thus, p53 is essential for preventing cellular transformation and the tumor development process. Therefore, regulation of p53 function is crucial.

Many cellular proteins regulate p53 function. The p53 protein level needs to be strictly controlled under different conditions. Under normal conditions, the p53 protein level is low and p53 is inactive, permitting cell proliferation. Under external stress, the p53 level is enhanced and p53 is activated, leading to the growth arrest or apoptosis of stressed and/or damaged cells. Several viral oncoproteins target p53. They need to inhibit the "cellular gatekeeper" function of p53 to allow for efficient viral infection. This can be achieved by enhancing ubiquitin-mediated degradation of p53, as is the case with SV40 large T antigen and HPV E6. The adenovirus E1B 55-kDa (E1B) protein does not degrade p53 but binds to and inactivates p53. The inactivation of p53 by E1B is thought to be a key step in cell transformation induced by adenovirus (Dix et al., 2000; Duque et al., 1999; Harada and Berk 1999). One mechanism by which E1B inhibits p53 activity

may be by interfering with the post-translational modification of the protein. We set out to determine whether E1B could interfere with acetylation of p53 by PCAF. Acetylation by PCAF enhances p53 sequence-specific DNA binding and regulates p53 nuclear retention.

E1B inhibits p53 acetylation by PCAF

In chapter 2, we show that E1B inhibits p53 acetylation by PCAF. E1B specifically inhibits p53 acetylation by PCAF in vitro (Fig. 2 of chapter 2) and in vivo (Fig. 3 of chapter 2) but not PCAF self-acetylation and histone acetylation. To explain how E1B specifically inhibits acetylation of p53 by PCAF, we tested the following possibilities: Is E1B a p53-specific deacetylase or does it recruit a deacetylase specific for p53? Is E1B a substrate of PCAF and does E1B compete with p53 for PCAF acetylation? Does E1B interfere with the interaction between PCAF and p53?

To study the possibility whether E1B is a p53-specific deacetylase or can recruit a deacetylase specific for p53, in vitro acetylation assays were performed. In this system, since only purified proteins are used, there should be no additional deacetylase. However, there is a small chance that during the protein purification process some non-specific proteins are eluted with E1B. The in vitro acetylation assay was performed by incubating p53, ^{14}C-acetyl-CoA and PCAF for 30 min and then adding E1B. If E1B is a p53-specific deacetylase or recruits a p53-specific deacetylase, it should decrease the amount of acetylated p53. However, we found that when p53 is acetylated before the addition of E1B, there is no change in the amount of acetylated p53 (data not shown). Therefore,

E1B is likely not acting as a deacetylase or recruiting a deacetylase. However, we cannot completely rule out this possibility because PCAF was not removed prior to the addition of E1B. The acetylase function of PCAF might abrogate the deacetylase function derived from E1B thus preventing p53 deacetylation. Interestingly, recent studies have shown that E1B interacts with histone deacetylase 1(HDAC1) and the transcriptional corepressor protein mSin3A. The E1B/mSin3A/HDAC1 complex is enzymatically active in catalyzing deacetylation of a histone substrate peptide (Punga and Akusjarvi 2000). Thus, it is possible that this complex might also play a role in inhibiting p53 acetylation in vivo and E1B might recruit other deacetylases in vivo.

To test the possibility whether E1B is an acetylation substrate of PCAF and competes with p53 to be acetylated by PCAF, we performed an in vitro acetylation assay. To do this E1B was incubated with PCAF along with ^{14}C-acetyl-CoA. As shown in Fig. 2A of chapter 2, E1B cannot be acetylated by PCAF in vitro.

To test the possibility that E1B interferes with the enzyme-substrate interaction between PCAF and p53, thereby inhibiting p53 acetylation by PCAF, immunoprecipitation and yeast two-hybrid assays were performed. PCAF binds to E1B and to a C terminal region of p53 encompassing lysine 320 (Fig. 7 and 8 of chapter 2), the specific PCAF acetylation site. We also show that p53 might interact with the central domain of PCAF, which contains the HAT domain and ADA2 domain (see our preliminary result in Fig. 6, chapter 4). Thus the interaction between PCAF and p53 might reflect enzyme-substrate interaction between the p53 region containing the acetylation site and the HAT domain of

PCAF. Since E1B binds to both p53 and PCAF, it is possible that the E1B-p53 and/or E1B-PCAF interactions interfere with the p53-PCAF interaction thus inhibiting p53 acetylation. In figure 9 of chapter 2, we show that an E1B mutant that failed to interact with PCAF was able to dissociate the p53-PCAF interaction in a reverse yeast two-hybrid assay. This implies that the binding of E1B to p53 is sufficient to interfere with the p53-PCAF interaction. Consistent with this, our preliminary data in chapter 4 indicate that E1B binds to the bromodomain of PCAF (Fig. 6) and also inhibits p53 acetylation by the bromodomain-deleted PCAF mutant (Fig. 7).

This leaves us with the question: what is the role of the E1B-PCAF interaction? The bromodomain is an approximately 110 amino acids module found in nearly all histone acetyltransferases (HATs) and the ATPase component of certain nucleosome remodelling complexes (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Jones et al., 2000; Pamblanco et al., 2001). The bromodomain is usually involved in protein-protein interactions (Dhalluin et al., 1999; Jones et al., 2000). For example, the bromodomain of CBP interacts with the ternary complex factor E1K-1 and facilitates the rapid transcriptional activation of c-fos (Nissen et al., 2001). The Zhou group showed that the bromodomain of PCAF binds to acetylated lysines of histones 3 and 4. The nature of the recognition of acetyl-lysines by the PCAF bromodomain is similar to that of acetyl-CoA by histone acetyl-transferase (Dhalluin et al., 1999). The Travers group found that the bromodomain of Gcn5p preferentially binds to an N terminal acetyl lysine residue of histone H4. It may discriminate between different acetylated lysine residues depending on the context in which they are displayed (Owen et al., 2000). Other studies showed that

p300 binds directly to chromatin and that the binding requires the p300 bromodomain (Manning et al., 2001; Polesskaya et al., 2001; Tomita et al., 2000). Loss of the bromodomain severely impaired p300's ability to activate the p21/WAF1/CIP1 promoter in transient reporter assays (Ohshima et al., 2001). These results suggest a critical role for the bromodomain in p300 functions. Recently, Harel-Bellan group showed that the myogenic factor, MyoD, binding to p300/CBP is enhanced when it is acetylated, and the interaction involves the bromodomain of CBP (Polesskaya et al., 2001). Considering these data, we suspected that the bromodomain of PCAF might also play a role in mediating the interaction between PCAF and chromatin through its interaction with acetyl lysine of histones 3 and 4 and/or the interaction with the acetyl lysine of non-histone target proteins such as p53. Therefore, the E1B-bromodomain interaction between E1B and PCAF might play a role in preventing PCAF from binding to chromatin or preventing PCAF from binding to acetylated p53 and/or other PCAF target non-histone proteins and inhibit PCAF function. One hypothesis is that, since PCAF bromodomain binds to the acetyl-lysine of a fragment from histone 3 it might also bind to acetyl-lysine of acetylated p53, though it failed to bind to the non-acetylated p53. If this is proven to be right, the E1B-PCAF interaction might compete with the interaction between the PCAF bromodomain and acetylated p53, thereby dissociating it. Although we do not know how important the postulated interaction between the PCAF bromodomain and acetylated p53 might be, we suspect it might play a role in regulating p53 transactivation function, stabilization and/or nuclear localization. Future studies designed to compare the binding ability of the PCAF bromodomain to non-acetylated p53 and acetylated p53 may perhaps answer this question.

The acetylation of p53 by PCAF is severely impaired in 293 cells expressing E1A and E1B proteins (Fig 3, chapter 2). There is a possibility that E1A also inhibits PCAF acetylase function on p53. Previous studies demonstrated that E1A binds to PCAF and may repress the acetylase activities of PCAF and p300 in vitro (Chakravarti et al., 1999, Hamamori et al., 1996), while other studies have shown that E1A stimulates the acetylase activity of p300/CBP under certain circumstances (Ait-Si-Ali et al., 1998). This underscores the importance of acetylation and the significance of interfering with p53 acetylation and acetylation of other cellular proteins by viral oncoproteins. Although the biological effects of acetylation of transcriptional activators are not entirely clear, acetylation has been shown to enhance protein-protein and/or protein-DNA interaction, regulate protein nuclear localization and protein stability. Thus, inhibition of p53 acetylation by E1B might lead to a reduction in p53 protein levels as well a reduction in the p53 coactivator recruitment ability, p53 DNA-binding ability and p53 nuclear retention. In fact, we have shown that in cells expressing E1B proteins, the ability of p53 to bind to a short, naked p53 consensus DNA binding site is reduced (Fig 4, chapter 2), indicating that E1B could affect p53 DNA binding. Future studies through immunoprecipitation assays and immunofluorescence assays could compare p53 coactivator recruitment ability and p53 nuclear retention in normal cells and in cells expressing E1B proteins in the presence or absence of transfected PCAF. This would tell us if E1B affects p53 coactivator recruitment ability and/or nuclear retention regulated by PCAF.

p73 is activated by PCAF

It was thought that p53 did not belong to a gene family until recently when two homologues of p53, p63 and p73 were identified. Like p53, both p63 and p73 can trigger cell cycle arrest and apoptosis. Thus, they may perhaps share some regulators with p53 in controlling cell growth.

Previous studies have shown that two cellular proteins, Mdm2 and p300/CBP, which bind to p53 and regulate p53 function also regulate p73 function. In chapter 3, we show that PCAF also plays a role in regulating p73 function. PCAF binds to a central domain of p73, which is shared by all p73 isoforms (derived from alternative splicing) and a small C-terminal region that is unique to p73α (Fig. 1 and 2 of chapter 3). Since the C-terminus of p73α is the specific PCAF acetylation domain, this interaction could also reflect substrate-enzyme interaction reminiscent of the interaction between p53 and PCAF. We determined that the PCAF specific acetylation site of p73α is lysine 623 (Fig. 3 of chapter 3). We further show that when this site is mutated to arginine, mutant p73α has lower activity in stimulating the transcription of target genes and in inducing cell growth arrest (Fig. 6). This suggests that PCAF acetylation of p73α contributes to p73α transactivation function. We also show that p73β, which lacks the unique C-terminus of p73α and was not acetylated by PCAF, is significantly activated by PCAF in a transient reporter assay (Fig. 4A, chapter 3). PCAF can stimulate p73-dependent apoptosis and reduce p73-dependent colony formation thus further demonstrating an important role for PCAF in stimulating p73 function.

As a transcriptional coactivator and acetyltransferase, PCAF has three main functions: HAT, FAT and activator binding function. Whether the HAT function of PCAF is required for regulating p53 and p73 is not yet clear. The fact that PCAF acetylates both p53 and p73α implies that the FAT function of PCAF is required to regulate p53, and p73α function. The p73 gene produces over six isoforms, the regulation of which may differ to some extent. For p73β and other p73 splicing variants, since they do not contain the unique small C-terminus (aa 560-636) of p73α encompassing the PCAF-specific acetylation site, the FAT function of PCAF may not play a direct role in regulating their function. However, we found that although PCAF failed to acetylate p73β, the acetylation domain (HAT domain) of PCAF is still required for PCAF to activate p73β-dependent transcription in a transient reporter assay (Fig. 4C, chapter 3). How can we explain this? There may be three possibilities: 1. The p73 binding region on PCAF is located in the HAT domain or close to the HAT domain, thus when the HAT is partly deleted (such as PCAFΔHAT1 and PCAFΔHAT2, Fig. 4C of chapter 3), the mutant PCAF is deficient in binding to p73 and in stimulating p73β function. In future studies, we could perform immunoprecipitation assays and/or yeast two-hybrid assays with p73 and PCAF deletion mutants, thus resolving the p73 binding domain on PCAF and addressing this question. 2. Though PCAF cannot acetylate p73β, it might need the HAT domain to exert its FAT function to acetylate other transcriptional factors which play critical roles in activating the transcription of p73β target gene. As more and more cellular factors involved in the p73 pathway are identified, this possibility could be

tested. 3. p73 might recruit PCAF for targeted nucleosomal acetylation of p73 target genes.

In trems of the activator binding function of PCAF, the interaction between PCAF and the p73α C-terminus might resemble the interaction between PCAF and p53, which reflects the enzyme-substrate interaction. The interaction between PCAF and p73 central domain might play a different and important role. In fact, while we show here that PCAF significantly stimulates p73β-dependent transcription in transient reporter assays (PG13-firefly luc as reporter), it failed to activate p53-dependent transcription in the same reporter system (unpublished data from our lab). This suggests that PCAF might serve more as a functional transcription coactivator of p73 than of p53 or it only exerts its coactivator function on p53 in response to DNA damage, when p53 acetylation is strongly stimulated. In summary, the three main functions of PCAF: HAT, FAT and activator binding function might all contribute to the regulation of both p53 and p73. However, they might not regulate p53 and p73 in the totally same way.

Liu et al. have reported that the full function of p53 requires both PCAF and p300. The two factors form a complex when bound to p53 and cooperatively activate p53 function (Liu et al., 1999). Recent publications by Zeng et al. (2000 and 2001) have shown that p300 also binds to p73 and serves as a coactivator of p73-dependent transcription. However, the HAT domain of p300 is not required in this process. Therefore, we suspect that PCAF might also form a complex with the p73 bound p300 to activate p73 function. While the acetylase function of p300 is not required, the acetylation function of PCAF might play a role in acetylating p73α and other factors as well as the nucleosomal

histones, thus activating p73 and other transcription factors. The acetylation function of PCAF might also promote the access of RNA polymerase II machinery to p73-bound DNA and activate p73 target gene transcription. Future studies with transient reporter assays may help us to observe the effect of PCAF on p300 function in activating p73 or the effect of p300 on PCAF function in activating p73.

Previous studies have shown that p53 acetylation plays a role in enhancing p53 sequence-specific DNA binding ability and in stabilizing p53 (Gu et al., 1997; Liu et al., 1999). A recent report indicated that the critical role of p53 acetylation is to promote coactivator recruitment and histone acetylation. p53 acetylation increases its association with coactivators such as CBP and TRRAP, which are also acetyltransferases, thus leading to enhanced histone acetylation and resulting in activation of p53-dependent transcription (Barlev et al., 2001). Here we show that when the PCAF-specific acetylation site of p73α is mutated (K623 to R), the mutant p73α is less efficient at activating target gene transcription and at reducing cell colony formation. Thus, p73α acetylation might also contribute to its transactivation function. Previous reports demonstrated that the small C-terminus of p73α serves as a negative regulator to inhibit p73α transactivation function. When the small C-terminus of p73α from amino acid 549 to 636 is deleted, p73α transcription function is enhanced (Ozaki et al., 1999). Furthermore, co-expression of the C-terminal portion of p73α with p73β resulted in reduced transcriptional activity of p73β (Ueda et al., 2001). Thus, acetylation of p73α at the C-terminal domain may play a role in relieving the inhibition and activate p73α. Future studies by including immunoprecipitation assays, EMSA and immunofluorescence assays could compare if

the wild type p73α and the mutant p73α (K623 to R) have the same coactivator recruitment ability, DNA-binding ability and nuclear retention. This would tell us how acetylation of p73α plays a role in activating p73α transactivation function.

Two other questions that are related to the results discussed in chapter 3 remain to be addressed. 1. How can we evaluate the importance of p73α acetylation apart from PCAF-p73α central domain interaction in stimulating p73α function? We have already observed the effect of acetylation on p73α function using the p73α acetylation deficient mutants. To address the importance of PCAF-p73α central domain interaction in activating p73α, we can construct a p73α deletion mutant lacking the PCAF binding domain. By comparing the function of this p73α mutant with that of p73α K623R and that of the wild type p73α, we can address this question. Besides, we can also construct a p73β mutant lacking the same region. This mutant will help us to prove the importance of PCAF-p73β interaction in stimulating p73β function. Because the central PCAF binding domain of p73 contains part of DBD and the whole OD, if it is deleted, p73 will not be able to bind to its consensus DNA sequence and form an oligomer and will not be able to activate transcription. Thus, before we make a PCAF binding mutant of p73, we should identify the PCAF binding sites of p73. While it may be possible to identify p73 mutants that only affect PCAF-binding but not other functions, the PCAF binding sites may overlap with p73 DBD or OD domain and thus such the p73 mutants may be deficient in DNA binding or oligomerization. In this case, we might be able to address the p73 binding domain on PCAF and make a PCAF mutant lacking p73 central domain binding function and test if this PCAF mutant can activate p73-dependent transcription. 2. Although we show that

PCAF further enhances p73α stimulated luc activity and further induces apoptosis and reduces colony formation when cotransfected with p73α and p73β (Fig. 4 and 5 in chapter 3), PCAF has an effect by itself. Can we rule out the possibility that the effects might be additive? For the transient reporter assay, we can try to use the same p73 mutant lacking PCAF binding domain as described above or try to find and use a new reporter plasmid that can be significantly activated by both p73α and p73β but not PCAF. For the apoptotic assay and colony formation assay, we can either try the same p73 mutant or perform the assays with p73 in a p53-PCAF double-deficient cell line. When the two remaining questions are answered, we will see a more complete picture of how PCAF regulates p73.

E1B does not affect PCAF function on p73

The emerging p73 network contains Mdm2, p300/CBP and PCAF, the same cellular proteins involved in the p53 signal transduction pathway. One may ask if the same set of viral proteins that inactivate p53 also inhibit p73 function, given the similar functions of both p53 and p73 in regulating cell growth arrest and apoptosis. For efficient infection, DNA viruses might also need to inhibit the function of p73. However, previous studies have shown that three main viral oncoproteins, Ad E1B-55kDa (E1B), HPV E6 and SV40 large T antigen, which bind to and inactivate p53 all failed to interact with and inactivate p73. In chapter 4, we show that E1B not only failed to inactivate p73α and p73 β-dependent transcription as reported by previous studies, but also failed to inhibit PCAF function in regulating p73 (Fig. 8). Furthermore, while E1B significantly inhibits p53 acetylation by PCAF, it has no effect on the acetylation of p73α C terminus (Fig. 8) by

PCAF. Then how can E1B distinctly regulate p53 and p73? A possible explanation is the failure for E1B to bind to p73. We have shown that though E1B binds to both p53 and PCAF, its interaction with p53 is sufficient for E1B to dissociate p53-PCAF interaction and to inhibit p53 acetylation by PCAF. A recent study by the Zhi-Min Yuan group showed

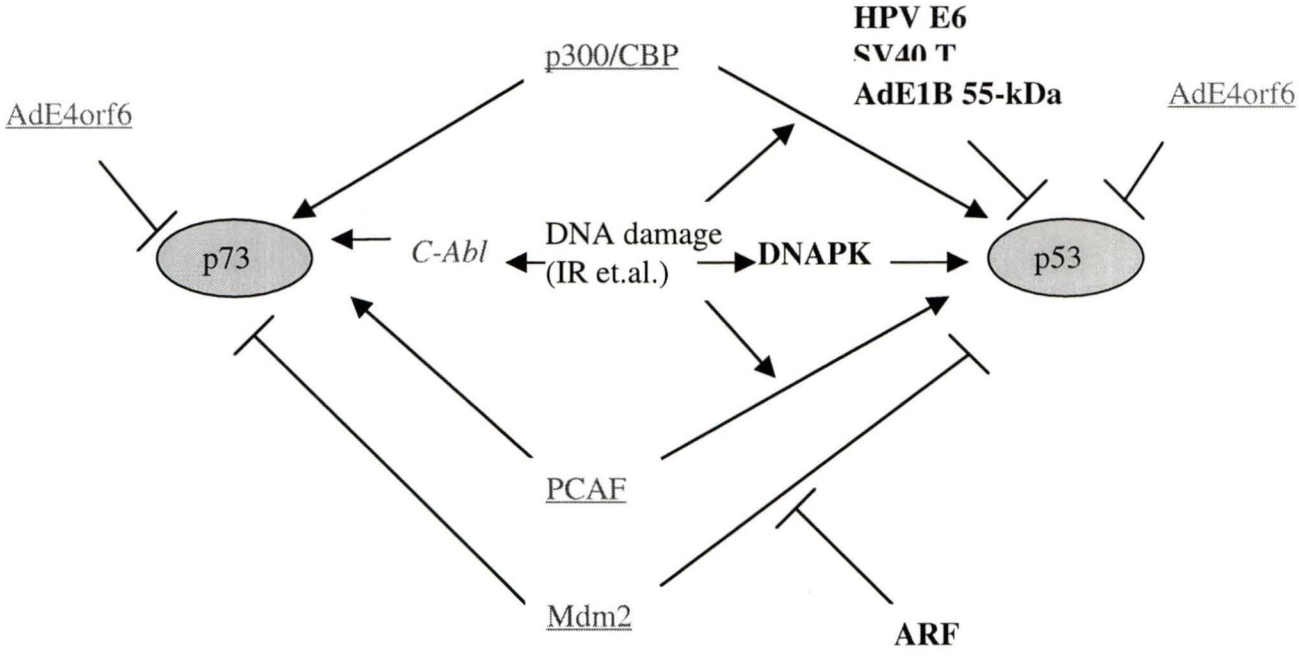

Fig 10. Signal transduction pathway involving p53 and p73. Proteins involved in both p53 and p73 networks are underlined. Proteins involved only in p53 pathway are in bold. Proteins involved only in p73 pathway are in italic. Cellular proteins p300/CBP, PCAF and Mdm2 regulate both p53 and p73 in a similar but distinct way. ARF inhibit Mdm3 E3 ligase function thus preventing Mdm2 to degrade p53. In response to DNA damage, p73 is activated by c-Abl-mediated tyrosine phosphorylation while p53 undergoes p300/CBP, PCAF-mediated acetylation and DNA-PK-mediated phosphorylation. Viral proteins distinctly regulate p53 and p73. Three viral oncoproteins, HPV E6, SV40 T and AdE1B 55-kDa that inactivate p53 all failed to inactivate p73. Adenovirus utilizes its E4orf6 oncoprotein to inhibit p73 function.

that a sequence element of p53 from amino acid 92-112 is required for viral proteins, including HPV E6 and E1B55 K/E4 34K, to target p53 to the proteolysis pathway (Gu et al., 2001). Since the same region (aa 92-112) of p73 is different from that of p53, E1B might fail to target p73 to the protein degradation pathway. Also, the interaction between E1B and PCAF might not dissociate the p73-PCAF interaction, which plays an important role to stimulate p73 transcription function. However, it might play other roles such as to regulate p73 stabilization and/or p73 protein distribution and nuclear retention. Future studies will address these issues.

Do other adenoviral proteins inactivate p73? Previous studies have shown that adenovirus utilizes the E4orf6 protein to inhibit p73-mediated transcription activation and cell growth arrest in a manner similar to its effect on p53. Recent studies showed that E1B is in complex with E4orf6, cullin 5, and Elongin B and C (personal communication with Dr. Arnie Berk). However, this complex can polyubiquitinate p53 but not p73 in vitro (Querido et al., 2001). Therefore, E4orf6 may utilize other ways to inhibit p73 function. This verifies again that the DNA viruses can utilize different viral proteins to cooperatively regulate the same set of cellular proteins with similar functions through multiple ways.

Conclusion

In conclusion, we studied the regulation of human tumor suppressor p53 and its family member p73 by the cellular regulator PCAF and the adenovirus oncoprotein E1B. We show that PCAF activates p73. Thus PCAF may be involved in the signal transduction

pathway of both p53 and p73. We also show that the adenovirus E1B oncoprotein can inhibit PCAF acetylation function on p53 but fails to affect PCAF function on p73. Inhibition of p53 acetylation by E1B leads to deficient protein-DNA binding between p53 and a short naked double-strand DNA fragment containing p53 consensus binding sites. This suggests a new mechanism for inactivation of p53 by E1B. We showed that E1B has no effect in inhibiting p73, confirming that adenovirus inactivates p53 and p73 in distinct ways. The research described in this thesis provides some insight into how the p53 family members exert their function, how PCAF activates p53 and its family member, and how DNA viral proteins interfere with the p53 family function on regulating their responsive genes. Research in this area will also help us to learn the function of non-histone protein acetylation and the function of transcriptional coactivators and acetylases. Furthermore, this research will help us to learn more about how adenovirus induces transformation and what the function of the E1B oncoprotein is. As more mysteries of adenoviruses are uncovered, humans will be able to develop more efficient ways to prevent and treat viral infections and design more efficient viral vectors for gene therapy.

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